EXPRESSION OF *SPIROPLASMA CITRI* PROTEINS UNDER VARYING *IN VITRO* CONDITIONS AND THEIR IMPORTANCE IN TRANSMISSION BY THE LEAFHOPPER *CIRCULIFER TENELLUS*

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CHAPTER I

INTRODUCTION

Members of the class Mollicutes are wall-less prokaryotes that are normally motile and pleomorphic. Cell morphology ranges from helical to non-helical filaments and coccoid cells (75). The term spiroplasma was coined in 1973 (22) to describe the subgroup of Mollicutes with helical shape. Motility and morphology are two major features which distinguish most spiroplasmas from other Mollicutes (68). However, Spiroplasma citri strain ASP-1 Townsend is non-helical and non-motile (67).

Spiroplasmas are surrounded by a trilaminar unit membrane which is exposed directly to the environment (19). Proteins on the surface of this membrane may be involved in host-pathogen interactions such as adherence, enzyme action, viral attachment, and pathogenicity, as demonstrated in some mycoplasma species (3, 12, 29, 34, 51, 52, 65). Twenty-nine different membrane proteins have been identified in S. citri Saglio, of which the amphiphilic protein spiralin is most prevalent (76, 79). Fletcher et al. (25) have identified eleven S. citri membrane proteins in addition to spiralin which have a surface component. The functions of the surface proteins remain unknown. ASP-1 lacks a protein (39

KDa) present in all other S. citri strains examined (67).

The goals of this project were to characterize the expression of S. citri proteins under varying in vitro conditions and to study their importance in transmission by the leafhopper Circulifer tenellus (Baker).

Specific objectives were:

(1) To characterize the production of proteins by S. citri strain BR3-3X under varying environmental conditions.

(2) To compare the protein profiles of several S. citri lines: (a) the non-helical strain ASP-1 with helical strains and (b) S. citri lines differing in transmission or subculturing history.

(3) To develop electron microscopy procedures necessary for later examination of the specificity of transmission of S. citri by the leafhopper C. tenellus: (a) to describe the morphology of the midgut of C. tenellus and (b) to specifically label S. citri within the embedding medium LR White.

These objectives may provide information related to the possible functions of spiroplasma proteins so that a better understanding of the mechanisms of spiroplasma morphology, pathogenicity, and transmissibility can be gained. This would provide information useful in the ultimate development of control methods.

CHAPTER II

LITERATURE REVIEW

Introduction

The Class Mollicutes consists of three families of cell wall-less, pleomorphic prokaryotes: Mycoplasmataceae, Acholeoplasmataceae, and Spiroplasmataceae (42). Mollicutes cannot synthesize peptidoglycan. Proteins comprise 57% of the total dry weight of the membrane, lipids 34%, and carbohydrates 2.2% (42). The Mollicutes were once thought to be filterable viruses, then bacterial L-forms, and later, one of the most primitive organisms known. They are now considered to be a branch of the low G+C gram-positive eubacteria (60). Based on sequence comparisons of rRNAs and tRNAs in recent phylogenetic studies, it appears that the Mollicutes arose through degenerative evolution from Clostridium spp. (Prazmowski) (41, 60, 78, 80).

The mycoplasma-like organisms (MLOs) are a group of organisms that as yet have not been definitively classified within the family Mycoplasmataceae. The plant pathogenic MLOs are prokaryotes that lack a cell wall, are pleomorphic, and have a low G+C content in their genome. These traits are similar to those of the class Mollicutes; but due to their fastidious nature, the MLOs have not yet been

characterized biochemically or nutritionally due to our inability to culture them in vitro (64). Sears et al. (64), suggest that MLOs may be more closely related to the acholeplasmas than to the mycoplasmas and spiroplasmas. Factors which support this possibility include the resistance of MLOs to digitonin treatments, suggesting that they do not require sterol for growth like acholeplasmas but unlike mycoplasmas and spiroplasmas. DNA sequence comparisons of the Oneothera aster yellows MLO and Acholeplasma laidlawii Edward and Freundt confirm that these two organisms are more closely related to each other than to mycoplasmas. Sears et al. (64) suggest that in the degenerative evolution of the three families of Mollicutes several distinct, different genomic losses have occurred.

Characteristics of the Family Spiroplasmataceae include helicity, motility without flagella (flexing, translational movement, and rotation about a longitudinal axis) (74), ability to live in two types of hosts (plant and insect), cholesterol requirement, and a genome molecular weight of approximately 10^9 daltons (61). The term "spiroplasma" was coined by Davis and Worley (22) to reflect the helical shape of these organisms. Motility and morphology are two major features which distinguish spiroplasmas from other Mollicutes (68). However, the way in which helicity and motility of spiroplasmas are maintained, despite the lack of flagella or cell wall, is unknown (67). As a spiroplasma

culture ages, helicity is lost and a variety of distorted forms are produced (19). Furthermore, some spiroplasmas lose helicity in the plant, such as S. kunkelii Whitcomb in broad bean (47);, or in the hemolymph of the insect vector, such as S. citri in Dalbulus elimatus (Ball), Macrosteles fascifrons (Stal) (77), and Euscelis plebejus (Fallen) (69).

Classification of spiroplasmas is based on the following tests: glucose fermentation, arginine catabolism, urea hydrolysis, temperature requirements, filtration characteristics, G+C content of DNA, and several serological tests (growth inhibition, metabolism inhibition, deformation) (71). Evaluation of the percent of comigrating and homologous proteins between spiroplasmas as seen in Western blots and polyacrylamide gels has been used to determine the relatedness of spiroplasmas (71).

Viruses are often associated with spiroplasmas. Three of these are SV-C1 (a rod-shaped virus), SV-C2 (a polyhedral virus with an isometric head and a long tail), and SV-C3 (a polyhedral virus with an isometric head containing linear dsDNA of approximately 21 kilobases and a short tail) (41, 48). A new virus, SpV4 (an isometric virion containing circular ssDNA of approximately 4400 bases), has recently been discovered in certain spiroplasmas (41, 48). Furthermore, many spiroplasmas have been shown to contain plasmids ranging from 2-50 kilobase pairs (5, 48).

In the 1960s MLOs were first associated with plant

diseases previously reported to have viral etiology, and with insects (72). Now, over 200 economically important diseases have been attributed to MLOs (36). They can be transmitted by grafting, parasitic plants (Cuscuta spp.), or arthropods (21). The association with arthropod vectors, which include the leafhoppers, planthoppers, and psyllids (43), is common among plant pathogenic spiroplasmas. Symptoms of MLO and spiroplasma induced disease in plants vary; but chlorosis, leaf mottling, proliferation of growing points, and stunting of growth are common (20, 72).

Spiroplasma citri

S. citri Saglio, described and named in 1973 (72), was the first spiroplasma to be cultured in vitro and the first for which Koch's postulates were fulfilled (20). S. citri cells are pleomorphic and vary in shape depending on the environment. The cells can be spherical or ovoid to helical, or branched, non-helical filaments. The average dimensions of log phase S. citri cells are 0.15-0.20 um in diameter and 2-5 um in length (20). They reproduce by binary fission (5). S. citri has a genome size of $1x10^9$ daltons and a G+C content of 26±1 mol%. It can pass through 0.22 um filters, is resistant to penicillin but sensitive to tetracycline, and is able to grow in agar, where it may produce "fried-egg" colonies (61). It may carry one or more plasmids or viruses. Two plasmids (pMH1 and pM41), two

bacteriophages, and one rod-shaped virus are among the extrachromosomal elements that have been found to be associated with S. citri (5, 42, 54) A rod-shaped virus is common in the plant pathogenic S. citri strains (23).

Life Cycle

S. citri has a wide geographical range. It is found throughout the United States (though most prevalent in Western states), North Africa, Mediterranean countries, Brazil, and Australia in warm to hot, dry regions. The host range of S. citri includes many species of dicots and monocots (8). Diseases caused by S. citri include citrus stubborn disease, horseradish brittleroot disease, and an unnamed disease of periwinkle (24). Until recently, S. citri and S. kunkelii, the corn stunt spiroplasma, were the only two spiroplasmas shown to induce diseases in plants (5, 62). A third species, S. phoeniceum (Saillard), was recently isolated from periwinkle in plants in Syria (62). The symptoms this spiroplasma induced, mainly yellowing, malformation of the leaves, rosetting, and dwarfing, resembled but were distinguishable from symptoms produced by S. citri (62).

S. citri has a complex life cycle involving multiplication in both insect and plant hosts. Transmission by the leafhopper Circulifer tenellus was detected in California and other areas in the late 60s and early 70s

(8). After S. citri is ingested by the leafhopper vector, it passes through the gut wall, multiplies in the hemocoel, and moves into the salivary glands from whence it can be inoculated into the phloem of a plant. Similarity in the phloem sieve tube contents and insect fluids could be a reason why both are suitable environments for S. citri (8, 72). S. citri can overwinter in leafhoppers, some herbaceous biennials and perennials, or some winter annuals (8).

The mechanisms by which S. citri causes disease are not completely understood, but the spiroplasma does interrupt the hormones controlling the plant's development and growth (42). The degree of mottling, stunting, phyllody, and wilting vary with host, environment, and spiroplasma strain (8). A major metabolic S. citri byproduct, lactic acid, might contribute to disease. Elevated levels of lactic acid have been detected in periwinkle plants infected by S. citri (20). Toxins may also contribute to the disease syndrome of mollicutes. A variety of mycoplasma toxins, including metabolic, diffusable compounds, endotoxin-like substances, and exotoxin-like substances, have been identified as being produced by mycoplasmas in culture and host tissues (70). However, definitive proof of a role for spiroplasma toxins in host plant metabolism is still lacking. Daniels (15) reported that S. citri cultures produce two toxins which might contribute to symptom severity. A polar, acidic toxin

(200-300 KDa) present in the leaves of broad bean plants infected with S. citri could not be purified due to instability, nor was it detected by gas liquid chromatography. A second toxin of S. citri was a low molecular weight neutral compound (17).

Control of mollicute-induced plant disease with insecticides is not effective because the leafhopper can transmit the spiroplasmas before the insecticide is effective. Weed control may actually enhance disease incidence by exposing susceptible crops previously protected by the weeds (8). Breeding for resistant plants, heat therapy, and antimycoplasma drugs have been effective in alleviating symptoms and/or controlling disease in certain cases. Proposed control methods include application of strain interference, introduction of spiroplasma-infecting viruses, use of antimetabolites, and production of spiroplasma mutants lethal to the vectors (8, 42).

Growth Requirements

To cultivate S. citri in vitro, long chain fatty acids and cholesterol must be provided since S. citri is unable to synthesize these materials (6, 10). The media best suited for growth of S. citri (C3G, M1, LD8, LD8A) contain animal serum, yeastolate, and PPLO (pleuropneumonialike organism) broth, which provide essential ingredients such as amino, organic, and fatty acids, sugars and cholesterol (6, 11,

36). Spiroplasmas within different serogroups vary in their utilization of carbohydrates (10).

S. citri has narrow ranges of optimal pH, temperature, and osmolarity. It has an optimal pH range of 7.4-7.6 and an optimal temperature range of 30-32 C (8). Its titer in the sieve tubes of plants is influenced by the ambient temperature of the host plant. Although growth is possible at higher and lower temperatures and pHs, cell morphology becomes distorted (8, 10, 11, 36). This distortion is due to secretion of significant amounts of acids by the S. citri cells during growth in the medium, which, if left unchecked, will have a lethal effect on the cells (11). S. citri is sensitive to osmotic shock, the optimal osmolarity ranging between 600-700 mOsms. Adjustments in the osmolarity of media can be made through increasing or decreasing sugars or salts. Both anaerobic and aerobic conditions are tolerated, though S. citri generally grows more rapidly in an anaerobic environment (5% carbon dioxide in nitrogen) (6, 36). Growth is most rapid in liquid media. As a culture ages, helicity is lost and a variety of forms is produced, including medusas (aggregates of helical cells), elongated cells, and coccoid cells (20, 72). Factors involved in in vitro cultivation may not be identical to those in the living host (11).

S. citri Membrane Proteins

S. citri cells are surrounded by a trilaminar unit membrane that is exposed directly to the environment (19). Proteins on the surface of this membrane may be involved in host-pathogen interactions similar to those described for zoopathogenic mycoplasmas, such as adherence, enzyme action, viral attachment, and pathogenicity (3, 12, 29, 34, 51, 52, 65). However, very little is known about the functions of spiroplasma membrane proteins.

Over 60 membrane proteins have been discovered in members of the family Spiroplasmataceae, the functions of which are unknown. Some of these membrane proteins have anomalous electrophoretic patterns, migrating different distances at different times under similar conditions (53). Foissac et al. (26) compared total protein profiles of eight S. citri strains by polyacrylamide gel electrophoresis (PAGE), noting anomalous behavior in the electrophoretic mobility of spiralin (29 KDa). Through protein analysis and amino acid sequencing, they determined that the mobility differences were not due to differences in size or modifications of the amino terminus of the protein. They are investigating whether amino acid modifications (especially acylation) might contribute to the anomalous behavior.

One dimensional PAGE separates polypeptides according to molecular mass, and two-dimensional PAGE enhances the

resolution by separating the proteins according to both isoelectric point and molecular mass (16). Through gel electrophoresis and Western blots, 29 different S. citri membrane proteins have been identified. The amphiphilic protein spiralin (a transmembrane protein) is the most prevalent and constitutes 22% of the total membrane protein (76, 79, 80). Fletcher et al. (25) used surface immunoprecipitation and protease techniques to identify twelve S. citri membrane proteins, including spiralin, which have a surface component. Spiralin and two other proteins (77KDa, 58KDa) were present in both pathogenic and non-pathogenic strains. However, two protein bands (86KDa, 89KDa) present in a pathogenic strain were missing in a nonpathogenic strain, indicating a possible role for the 86/89 KDa proteins in pathogenesis.

Objective I: Differential Expression of Proteins by Microorganisms Grown Under Varying Environmental Conditions

The first objective of this project was to characterize the expression of S. citri surface proteins by varying environmental conditions in vitro. There is precedence for the differential expression of microbial membrane proteins under varying environmental conditions. In Escherichia coli (Migula), Omp T gene expression is influenced by temperature (58), while Omp F and Omp C are pH-dependent (32, 58).

Transcription of other surface proteins is influenced by osmolarity (58, 59). However, the presence or absence of oxygen did not produce any noticable changes in E. coli protein patterns (58).

The effects of osmolarity were examined in Agrobacterium tumefaciens (Smith and Towsend) mutants that lack periplasmic beta-1,2-glucan and exhibit pleiotropic phenotypes. This glucan maintains a high osmolarity environment for the bacterium in the presence of low osmotic strength media. It has been suggested that the glucan has an effect on attachment, virulence, and motility of the bacterium (9). Though differences were noted in cytoplasmic and periplasmic protein content and glucan synthesis, these were not correlated with virulence or motility. Thus, glucan function is not related to osmoadaptation, but is possibly related to virulence, attachment, and motility (9).

In Pseudomonas (Migula) species, certain outer membrane proteins are affected by variations in the environment (31). Iron-repressible outer membrane proteins, which function as receptors for the binding of complexes of iron with siderphores, are produced when iron is lacking. OprP, a protein important in the high-affinity, phosphate starvation inducible, phosphate specific transport system, is produced only in low phosphate conditions. OprF, an outer membrane protein possibly involved in the formation of channels allowing passage of saccharides (3000 MW), is affected by

variances in osmolarity. OprG, a protein possibly involved in fluoroquinolone uptake and/or low affinity iron uptake, is dependent on growth conditions. Finally, OprH, a protein which replaces divalent cations in the outer membrane and blocks self-promoted uptake of polycationic antibiotics, is overexpressed when cells are grown in media lacking magnesium, calcium, and manganese (31).

The effect of pH on the growth rate of a strain of Bacteriodes intermedius (Johnson and Holdeman), a bacterium possibly involved in periodontal disease, was investigated as a means of control. The production of outer membrane proteins and antigenic profiles were stable in different pH; although there was variation in the amounts of the proteins synthesized (7).

Brown spot disease of bush beans is caused by Pseudomonas syringae pv. syringae strain R32 (Rudolph). Through comparisons of the pathogenic strain with the Tn5 derived, non-pathogenic P.s.pv.s. strain PS9021, several surface proteins of Pseudomonas were found to be associated with pathogenicity. Strain R32-specific antiserum, cross-absorbed with a PS9021 protein preparation, retained activity against the pathogenic strain but not the non-pathogenic strain, indicating that pathogenicity-specific proteins were present in strain R32. Cultivation of cells grown at temperatures $\geq$ 30 C resulted in a loss of the pathogenicity specific proteins, thus suggesting

temperature-dependent regulation. Disease usually resulted at low temperatures and high humidity (57).

Effects of heat shock on three mollicutes (Acholeplasma laidlawii K2 and JA1 and Mycoplasma capricolum (Tully) Kid) were described. Significant increases in the synthesis of certain proteins (66-68 and 26-29 KDa) indicated induction of these genes under heat shock conditions (42 C). The presence of a heat shock system in these mollicutes suggests the importance of this system in the cell physiology of certain mycoplasmas (18).

The membrane surface potential of spiroplasmas is believed to be important in such processes as cell to cell adhesion, membrane fusion, and ion transport (63). The lipid phosphate groups of Spiroplasma floricola Clark cover the outer membrane surface and induce a negative membrane surface potential. Differences in the membrane potential of S. floricola were noted when the organism was subjected to varying pH and NaCl concentrations, although varying cholesterol and treating with pronase did not (63).

Fungi, too, are affected by variance in environmental factors such as temperature, pH, osmotic pressure, and nutrition. Many fungi require a narrow pH range, broad osmolarity and temperature ranges, and a carbon-nitrogen balance before fructification will occur (56).

Objective II: Protein Profiles of S. citri Lines

The second objective was to characterize the protein profiles of S. citri strain ASP-1 Townsend and four experimental lines that differed in transmission and subculturing history.

Spiroplasma citri strain ASP-1

Identified as a spiroplasma through a battery of tests ranging from serology to pathogenicity, ASP-1 is a unique S. citri strain that is both non-helical and non-motile (67). Previous work demonstrated that this strain produced symptoms in plants identical to those in plants infected by helical S. citri strains (68); however, recent pathogenicity tests with ASP-1 have been negative (C.J. Chang, personal communication). All three types of spiroplasma-associated viruses, SV-C1, SV-C2, and SV-C3, were identified in ASP-1. Optimal growth temperature is 32 C; and varying osmolarity does not induce any differences in morphology. ASP-1 has a longer lag phase in culture than most helical strains. It produces transparent, smooth, well-defined "fried egg" colonies without satellite colonies on agar, compared to helical strains which produce more diffuse "fried egg" colonies with satellite colonies. In liquid culture ASP-1 has a tendency to form aggregates.

ASP-1 lacks the 39KDa protein present in all other S. citri strains examined by gel electrophoresis (67). A protein missing in this strain compared to helical strains

could indicate its role in helicity and/or motility which are two major characteristics used in distinguishing spiroplasmas from other Mollicutes (33). ASP-1 has until recently been the only known exception. However, Lee and Davis (37) isolated both non-helical and partially-helical mutants of S. kunkelii, suggesting that this parameter of classification may not be entirely appropriate.

Experimental S. citri Lines

The four lines of S. citri strain BR3 differing in transmission and subculturing history are designated BR3-T, BR3-M, BR3-P, and BR3-G (4, 73). BR3-T is a line maintained by leafhopper (Circulifer tenellus (Baker)) transmission in turnip plants in the greenhouse. BR3-M is a line resulting from 41 serial passages of BR3 in liquid medium (insect transmissibility and pathogenicity have not been tested). BR3-P has been subcultured 132 passages (plants exposed to leafhoppers microinjected with BR3-P have not become diseased). BR3-G is a line that has been graft transmitted for eight years in periwinkle (causes symptoms on periwinkle but is no longer insect transmissible).

Certain zoopathogenic mycoplasma species have specific surface proteins demonstrated to function in recognition of host cells, attachment, and enzyme activities (3, 12, 29, 34, 51, 52, 65). The four experimental lines appear to differ in several proteins, and it is possible that one or

more of the proteins differentially expressed in these four lines are involved in similar activities related to pathogenicity or insect transmissibility. Further characterization of such proteins may contribute to the knowledge of the determinants of these critical events.

Spiroplasma Transmission

Little is known about the molecular interactions of phytopathogenic mollicutes and their leafhopper vectors. Plant virologists have shown that specificity in transmission of circulative plant viruses by aphids is dependent on recognition and binding of a virus coat protein to a specific receptor on the aphid gut wall (27, 28). Thus, recognition may also be involved in leafhopper transmission of spiroplasmas and this question has been approached using electron microscopy.

The goal of the third objective was to develop electron microscopy procedures necessary for later examination of the specficity of transmission of S. citri by the leafhopper C. tenellus by (a) describing the morphology of the midgut of C. tenellus and (b) specifically labelling S. citri within the embedding medium LR White. This preliminary work will lay the foundation for future research in which the questions of how spiroplasmas traverse the physical barriers within the insect (gut wall and salivary glands), and whether it is pathogenicity and/or transmissibility of

spiroplasmas that is lost as they are repeatedly passed both *in vivo* and *in vitro* can be answered.

Circulifer tenellus

Transmission of spiroplasmas by leafhoppers is a complex biological process. Once ingested by the leafhopper, a spiroplasma must pass through the gut wall, hemocoel, and salivary glands in order to multiply and complete its disease cycle (72). This type of transmission, in which the pathogen circulates and replicates within the vector, is propagative.

The major vector of *S. citri* is the beet leafhopper, *C. tenellus*. The life cycle of this insect usually includes two to three generations per year. Females are fertilized in the fall and the adult leafhoppers overwinter on weed hosts. In late winter and early spring 300-400 eggs per female are deposited on plant leaves and stems and hatching is temperature dependent. As soon as the nymphs hatch, they can feed and aquire spiroplasmas. Maturation is complete in 3-6 weeks, after five successive molts (8). Liu (38, 39) determined a minimum aquisition access period for *S. citri* of six days by *C. tenellus* while latent periods ranged from 10 days (injected insects) to 16 days (plant-fed insects), to 24 days (membrane-fed insects).

S. citri is pathogenic to *C. tenellus* (40). Infections can reduce fecundity, retard growth, and cause death of male

zygotes and premature death of infected leafhoppers (46, 50). Upon transmission to the plant, the spiroplasmas are systemically distributed in the phloem due to passive flow with the photosynthates that are transported to the plant's growing points (49). There appears to be a correlation between symptom severity and the number of spiroplasma cells present (20).

Leafhopper Anatomy of Alimentary Canal and Salivary Glands

The digestive tract of leafhoppers, specifically the family Cicadellidae of which C. tenellus is a member, is composed of the maxillary stylets, precibarium, and gut (foregut, midgut, hindgut). Fluid is sucked up through the stylets by cibarial pump action. The fluid then passes through the cibarium into the esophagus which opens into the midgut. Located within the anterior midgut is a filter chamber which shunts water entering from the foregut directly to the hindgut, thus concentrating the nutrients. Malpighian tubules are joined to the posterior end of the midgut allowing waste products to enter the hindgut (2).

The midgut is of endodermal origin unlike the foregut and hindgut which are ectodermal in origin. Ultrastructurally, the outside of the midgut is surrounded by a basal membrane followed by an outer muscular layer, containing different muscle types. Beneath these muscles

follows an epithelial layer of cells of varying shapes, normally binucleate and having a striated brush border. Closely packed, double or multimembraned microvilli surrounded this border. Occasionally, smaller cells called nidi, replacement, or regenerative cells are found at the base of the epithelial cells, under the basal membrane (2).

Leafhopper salivary glands are positioned between the head and thorax. A principal gland and an accessory gland located on either side of the longitudinal axis are both composed of secretory and duct cells. The common salivary duct is formed by the union of the glands and discharges saliva into the salivary pump, which is connected to the salivary canal (2).

Mechanisms of Spiroplasma Penetration of Leafhopper Anatomical Barriers

The mechanism of penetration of the gut epithelium and the salivary glands by *S. citri* remain unknown. Liu (38) proposed direct penetration and penetration via the endoplasmic reticulum as the means by which *S. citri* traverses the gut wall epithelial cells and the salivary glands of the leafhopper *C. tenellus*. He suggested that after *C. tenellus* ingests *S. citri*, some of the spiroplasmas move into the gut epithelial cells and multiply intracellularly, while others move across the basal membrane, enter the hemocoel, and multiply there. Finally,

the spiroplasmas invade the salivary glands, where multiplication continues. Liu noted that the spiroplasmas were grouped in bunches around the periphery of the acini (lobes) of the glands, and that the spiroplasmas lost their helical morphology and assumed the shape of a "main body" with long thin extensions (38).

Markham and Alivizatos (45) observed S. kunkelii near the periphery of the glands of the vector leafhopper Dalbulus maidis, always between the cell membrane and an outer membrane, suggesting intercellular passage to the salivary ducts. In later work Markham (44) observed large numbers of S. kunkelii grouped together along the acini periphery, bound by the plasmalemma on the inside and another membrane on the outside. He postulated that S. kunkelii enters the salivary glands (as well as the gut epithelium) via cell junctions. Alivizatos (1) also noted membrane bound S. kunkelii near the acini periphery of the glands of the leafhopper Euscelidius variegatus (Kirschbaum).

Mowry (55) determined that the leafhopper Macrosteles fascifrons (Stal) (a "poor" vector of S. citri) imposed a variety of barriers (pre-intestinal and post-intestinal) to the spiroplasma which the spiroplasma seems to penetrate by endocytosis. Hackett and Clark (30) stated that evidence is still inconclusive whether spiroplasma penetration is by endocytosis or diacytosis (direct penetration).

Other insect/pathogen systems may provide a clue. The mechanism of barley yellow dwarf virus (BYDV) penetration of the aphid Rhopalosiphum padi L. was described as endocytosis based on electron microscopy. Virions were transported from the hindgut to the hemocoel by specific fusion of the virions with the basal membrane. Gildow (27) inferred that the same mechanism of penetration occurred in the salivary glands. In further work, Gildow (28) proposed that the viruses move by receptor-mediated endocytosis through the hindgut epithelial plasmalemma of the aphids. In both of the previous examples the viruses were ingested, travelled to the hindgut, entered the hemocoel, and passed into the accessory salivary glands and duct. This route of vector infection may be similar to leafhopper infection by S. citri.

Recognition and Binding of Animal and Human Mycoplasmas

The single membrane surrounding mycoplasmas may allow the organisms to adapt to the host environment through attachment at the cell surface and possibly alter host cell functions (81) which could lead to disease. For example, in the case of Mycoplasma pneumoniae Somerson, which causes primary atypical pneumonia in humans, surface adhesion proteins designated P1 and P30 have been implicated in adherence of the mycoplasma to respiratory epithelial cells

(13). The P1 protein is similar to a protein of M. genitalium Tully, the pathogen causing nongonococcal urethritis, that is involved in adherence to the urinary tract (14). Both of these organisms require the adhesin proteins for binding and recognition. It is possible that such proteins exist in spiroplasmas and that these proteins are involved in attachment within the vector.

Transmissibility Versus Pathogenicity

Frequent and numerous passing of spiroplasmas in culture medium may result in the loss of either pathogenicity or transmissibility (8). Liu et al. (39), studying transmission characteristics of S. citri MV101 (isolated from naturally infected periwinkle), noted that the lines subcultured more than five times and injected into C. tenellus were not transmitted. However, S. citri strain Palmyra Fos (cultured from Circulifer haematoceps (Mulsant and Rey)) was still transmitted to plants after 65 transfers in culture medium (8).

Summary

These three objectives interrelate to provide information related to the possible functions of spiroplasma proteins. The first objective was to characterize the expression of S. citri surface proteins under varying culture conditions. Precedence exists for differential

expression of microbial membrane proteins under varying environmental conditions.

The second objective involved comparing proteins from different S. citri lines. Proteins missing in the non-helical mutant could play a role in helicity and/or motility of spiroplasmas, and proteins differing in the four experimental lines could play a role in insect transmissibility and/or pathogenicity.

The third objective involved electron microscopy examination of S. citri and its principal vector, the leafhopper C. tenellus. The knowledge obtained should promote a better understanding of the interactions between plant mollicutes and their insect hosts.

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CHAPTER III

Production of *Spiroplasma citri* Proteins Under Varying Environmental Conditions

ABSTRACT

Spiroplasmas, members of the class Mollicutes, are surrounded only by a trilaminar unit membrane (6). Proteins on the membrane surface of spiroplasmas, as in some mycoplasma species (1,4,11,14,17,18,24), may be involved in host-pathogen interactions such as adherence, enzyme activity, viral attachment, or pathogenicity. Twenty-nine different membrane proteins have been identified in *S. citri* (25), but the functions of these proteins are unknown (19). Production of proteins by *Spiroplasma citri* strain BR3-3x, was assessed under varying environmental conditions including pH, osmolarity, temperature, and oxygen availability. Proteins were assayed by one dimensional polyacrylamide gel electrophoresis and Western blotting, using antiserum to whole cells or to specific *S. citri* proteins. No differences were detected in the protein profiles of cells grown under these conditions.

INTRODUCTION

Spiroplasma citri Saglio causes a variety of diseases

including citrus stubborn disease, horseradish brittleroot disease, and an unnamed disease of periwinkle (8). It has a complex life cycle involving passage in two hosts, an insect and a plant (6). Numerous nutritional and environmental requirements must be met for the cultivation of S. citri in vitro. It has fairly narrow permissive ranges of pH (7.4-7.6), osmolarity (600-700 mOsms), and temperature (30-32). Both anaerobic and aerobic conditions are tolerated (16).

Since spiroplasmas lack cell walls, they are surrounded only by a trilaminar unit membrane that is exposed directly to the environment (6). Through gel electrophoresis and Western blots, 29 different S. citri membrane proteins have been identified (25), at least twelve of which have surface components (11). Although the functions of the spiroplasma surface proteins are unknown (19), surface proteins of some mycoplasma species have been implicated in host-pathogen interactions (1,4,11,14,17,18,24). The purpose of this study was to characterize the electrophoretic protein profiles of S. citri strain BR3-3x grown under varying environmental conditions.

MATERIALS AND METHODS

Cell preparation. All experiments were carried out using S. citri strain BR3-3x, originally isolated from Illinois horseradish affected with brittle root disease (8) and subcloned three times. Cultures of BR3, serially passed

28 times in liquid medium, were exposed to growth conditions reported to be optimal for S. citri (pH 7.5, 650 mOsms, 31 C, aerobic environment) (16) except that one of the following environmental conditions was varied on either side of the optimum: pH (7.00,7.25,7.50,7.75, 8.00), osmolarity (600,625,650,675,700 mOsms), temperature (26,27,28,29,30,31, 32,33,34,35 C), and oxygen availability (anaerobic versus aerobic; anaerobic conditions were achieved by incubating cultures in a GasPak System, BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD).

Within each of the environmental conditions tested (except oxygen availability), two types of protein comparisons were assessed: one in which cells were collected as each treatment reached log phase, and the other where all treatments were harvested when the culture grown under the optimal condition (center treatment) reached log phase.

Cell collection. LD8 broth medium (15) was inoculated with S. citri at an initial concentration of $2x10^5$ cells/ml. Log phase was determined by visual inspection: small, tight helices with 3 to 4 turns and a cell count of approximately 10^8 cells/ml. After cultures reached log phase, cells were harvested by centrifugation at 10,000xg for 30 min at 4 C. The pellets were washed twice in N-2-hydroxyethyl-piperazine-N'-2- ethanesulfanic acid, pH 7.5, with 10% sucrose (HEPES-S) and each pellet was resuspended in two volumes of HEPES-S. The mixtures were sonicated (Model W-

385 sonicator, Heat Systems Ultrasonics, Inc., Farmingdale, NY) using a continuous pulse at 30% duty cycle and ≤ 20% output power, for a few seconds to disperse cell clumps, then frozen at -20 C.

Protein concentration standardization. Protein concentrations of all preparations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using lyophilized bovine gamma globulin as the protein standard and a medium control. Concentrations of each sample were adjusted to match that of the protein sample with the lowest concentration.

PAGE. For polyacrylamide gel electrophoresis (PAGE) and Western blotting, protein concentrations were adjusted to 10 ug per sample (Bio-Rad Laboratories, Richmond, CA). Protein samples were mixed 1:1 with sample buffer (0.06M Tris-HCl, 10% glycerol, 1.5mM Bromophenol blue, 2% SDS, 0.26M beta-mercaptoethanol), boiled for 5 min, and loaded into wells of one dimensional 10% polyacrylamide gels. Electrophoresis was carried out according to the procedure of Laemmli (1970) in 1.5 mm slab gels. Gels were silver stained (7) or were electrophoretically transferred to nitrocellulose for Western blotting.

Western blotting. Proteins from polyacrylamide gels were electrophoretically transferred to nitrocellulose membranes in transfer buffer (192 mM glycine, 25 mM Tris pH 8.3, and 20% vol/vol methanol) using a Transphor apparatus

(Bio-Rad, Richmond, California) at 100 volts for 1.25 hr. The nitrocellulose membranes containing the transferred proteins were blocked overnight at 4 C in skim milk (5% in TBS (20 mM Tris, 500 mM NaCl, pH 7.5)). Membranes were washed three times with TBS, and incubated at room temperature for three hr with agitation with antiserum specific for total cell proteins, or with a mixture of antisera specific for four proteins having membrane surface epitopes (P89, P78, P55, P29) (10) diluted 1:100 in HEPES-S. The nitrocellulose was washed twice in TTBS (TBS containing Tween-20), once in TBS, and incubated for one hr with agitation in goat anti-rabbit IgG peroxidase conjugate (GARP, Sigma Chemical Co., St. Louis, Missouri), diluted 1:10 in TBS. After washing the membranes three times with TBS, the enzyme substrate, 1 part 4-chloro-1-naphthol (3 mg/ml in methanol, Sigma Chemical Co.), 5 parts TBS, and 0.018 part of 3% hydrogen peroxide, was added. Maximal color developed in 1-2 min. Blots were washed in water and air-dried.

RESULTS

pH. Protein profiles of S. citri cell cultures grown at different pHs within the range of 7.0-8.0 revealed no differences. This was true for both the whole protein profiles (Figures 1 and 2) and the four surface proteins for which specific antiserum was used (Figure 3). As the

cultures aged, the pH of the medium became increasingly acidic. A culture inoculated at pH 8.0 measured 7.7 at log phase, while one inoculated at pH 7.5 measured 7.2 at log phase. The cells became distorted in appearance over time.

Osmolarity. PAGE profiles of S. citri cell cultures grown at different osmolarities within the range of 600-700 mOsms revealed no differences (Figure 4). This was also true for Western blots developed with anti-whole cell serum or with a mixture of sera specific for four surface proteins.

Temperature. Protein profiles of S. citri cell cultures grown at different temperatures within the range of 26-35 C revealed no differences (Figure 5). Furthermore, no differences in the protein profiles were detected on Western blots developed with anti-whole cell serum or with a mixture of sera specific for four surface proteins.

Oxygen availability. Protein profiles of S. citri cell cultures grown in either anaerobic or aerobic conditions revealed no differences (Figure 6). Blots developed with anti-whole cell serum or with a mixture of sera specific for four surface proteins also showed no differences in the protein profiles.

DISCUSSION

Differential production of proteins in mollicutes and E. coli may occur when cells are subjected to altered

environments (2,3,5,12,13,21,22,23). S. citri has fairly narrow pH, osmolarity, and temperature ranges. Cell characteristics, growth rate, and titer are determined by the initial pH, osmolarity, and temperature (16). Deviations from these optimal ranges may result in aberrant cell forms.

If protein profiles of S. citri were affected by varying environmental growth conditions, this information might be useful in identifying possible roles for these proteins and eventually in the control of this pathogen as in other systems. For example, through comparisons of a pathogenic strain of Pseudomonas syringae pv. syringae, R32, with a Tn5 derived non-pathogenic strain, PS9021, surface proteins of Pseudomonas were found to be associated with pathogenicity. Strain R32-specific antiserum, cross-absorbed with a PS9021 protein preparation, retained activity against the former, but not the latter, indicating that pathogenicity-specific proteins were present in strain R32. Cultivation of cells grown at temperatures $\geq$ 30 C resulted in loss of pathogenicity-specific proteins as well as less severe symptoms in host plants, thus suggesting temperature-dependent regulation (20).

In E. coli, Omp T gene expression is influenced by temperature (21), while Omp F and Omp C are pH-dependent (13,21). Transcription of other surface proteins is influenced by osmolarity (21,22).

Certain outer membrane proteins of Pseudomonas species are affected by the presence or absence of substrates in the environment. Proteins such as OprP (a protein important in the high-affinity, phosphate starvation inducible, phosphate specific transport system) and iron repressible outer membrane proteins (proteins which function as receptors for the binding of complexes of iron with siderphores) are produced when their substrates are absent (iron) or in low concentrations (phosphate) thereby allowing the organisms to survive (12).

At present, the functions of S. citri membrane proteins are unknown (19). It was hoped that by varying the environmental conditions of S. citri in vitro and assessing the resulting protein composition, insight could be gained into how this organism survives and adapts to its environment. For example, if additional proteins had been observed when the temperature was varied, these proteins could be temperature-dependent, thus allowing S. citri to adapt to the fluctuating environment. However, in these experiments, no differences were detected in the protein composition of spiroplasmas grown under varying conditions. Perhaps other methods, such as capillary electrophoresis and chromatography could be used to yield some insight into the function of these proteins.

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Fig. 1. Silver-stained SDS-PAGE gel of total protein profiles of S. citri cell cultures grown within the pH range of 7.0-8.0. Lanes A,F (pH 7.00), lanes B,G (pH 7.25), lanes C,H (pH 7.50), lanes D,I (pH 7.75), lanes E,J (pH 8.00), lane S (molecular weight standard). Lanes A-E (all cultures allowed to reach log phase); lanes F-J (all cultures harvested simultaneously, when the lane H culture had reached log phase). Numbers on the right represent the protein sizes of the standard in KDa.

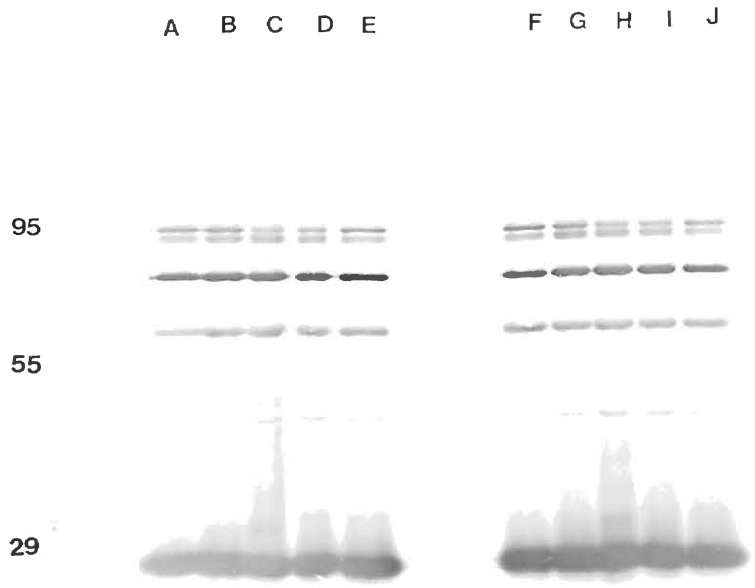

Fig. 2. Western blot of total protein profiles of S. citri cell cultures grown within the pH range of 7.0-8.0 developed with anti-whole cell serum. Lanes A,F (pH 7.0), lanes B,G (pH 7.25), lanes C,H (pH 7.50), lanes D,I (pH 7.75), lanes E,J (pH 8.00). Lanes A-E (all cultures allowed to reach log phase); lanes F-J (all cultures harvested simultaneously, when the lane H culture had reached log phase). Numbers on the right represent the protein sizes of the standard in KDa.

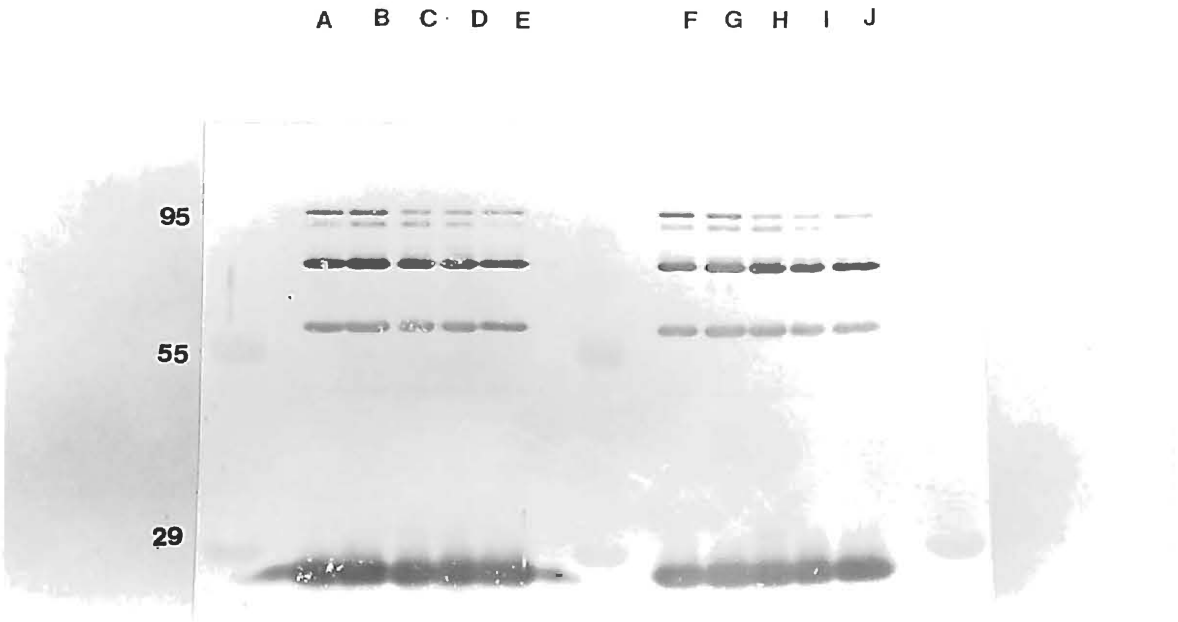

Fig. 3. Western blot of total protein profiles of S. citri cell cultures grown within the pH range of 7.0-8.0 developed with a mixture of p89, p78, p55, p29 antisera. Lanes A,F (pH 7.00), lanes B,G (pH 7.25), lanes C,H (pH 7.50), lanes D,I (pH 7.75), lanes E,J (pH 8.00). Lanes A-E (all cultures allowed to reach log phase); lanes F-J (all cultures harvested simultaneously, when the lane H culture had reached log phase). Numbers on the right represent the protein sizes of the standard in KDa.

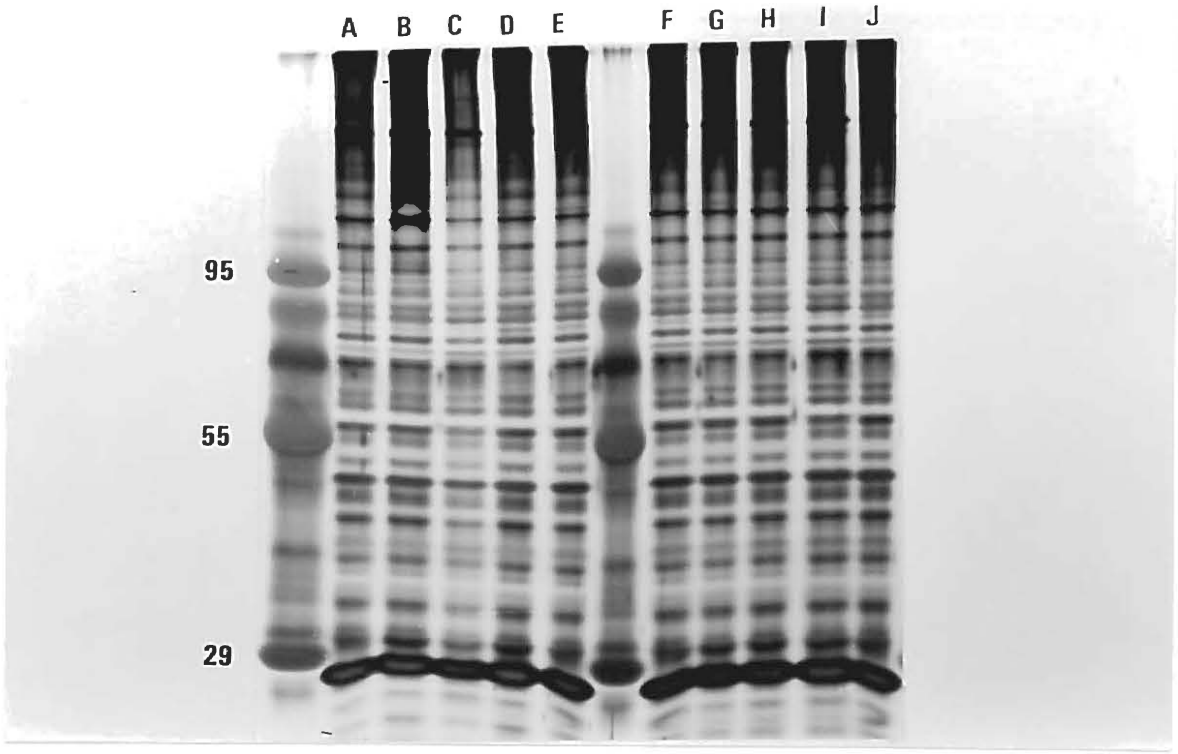

Fig. 4. Silver-stained SDS-PAGE gel of total protein profiles of S. citri cell cultures grown within the osmolarity range of 600-700 mOsms. Lanes A,F (600 Osms), lanes B,G (625 Osms), lanes C,H (650 Osms), lanes D,I (675 Osms), lanes E,J (700 Osms). Lanes A-E (all cultures allowed to reach log phase); lanes F-J (all cultures harvested simultaneously, when the lane H culture had reached log phase). Numbers on the right represent protein sizes of the standard in KDa.

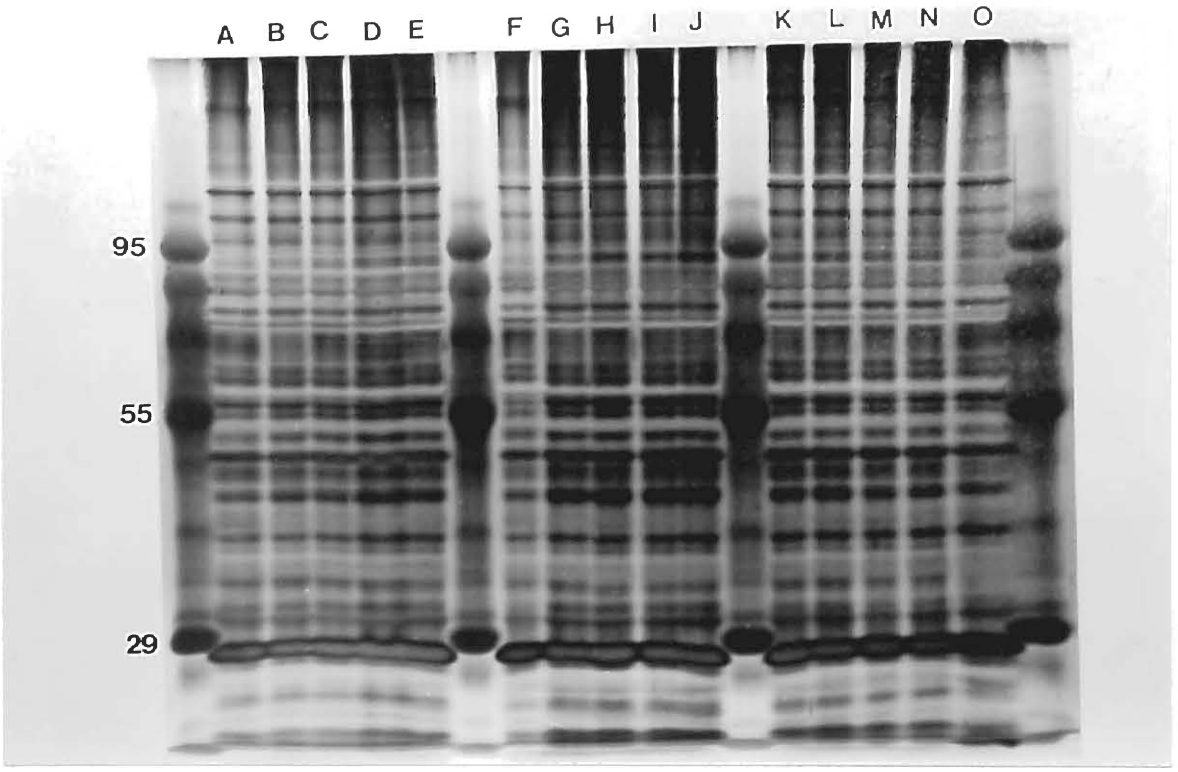

Fig. 5. Silver-stained SDS-PAGE gel of total protein profiles of S. citri cell cultures grown within the temperature range 26-35 C. Lanes A-E (26-30 C), lanes F-G (28-33 C), lanes K-O (31-35 C). Lanes A,E and K-O (all cultures allowed to reach log phase); lanes F-J (all cultures harvested simultaneously, when the lane H culture had reached log phase). Numbers on the right represent the protein sizes of the standard in KDa.

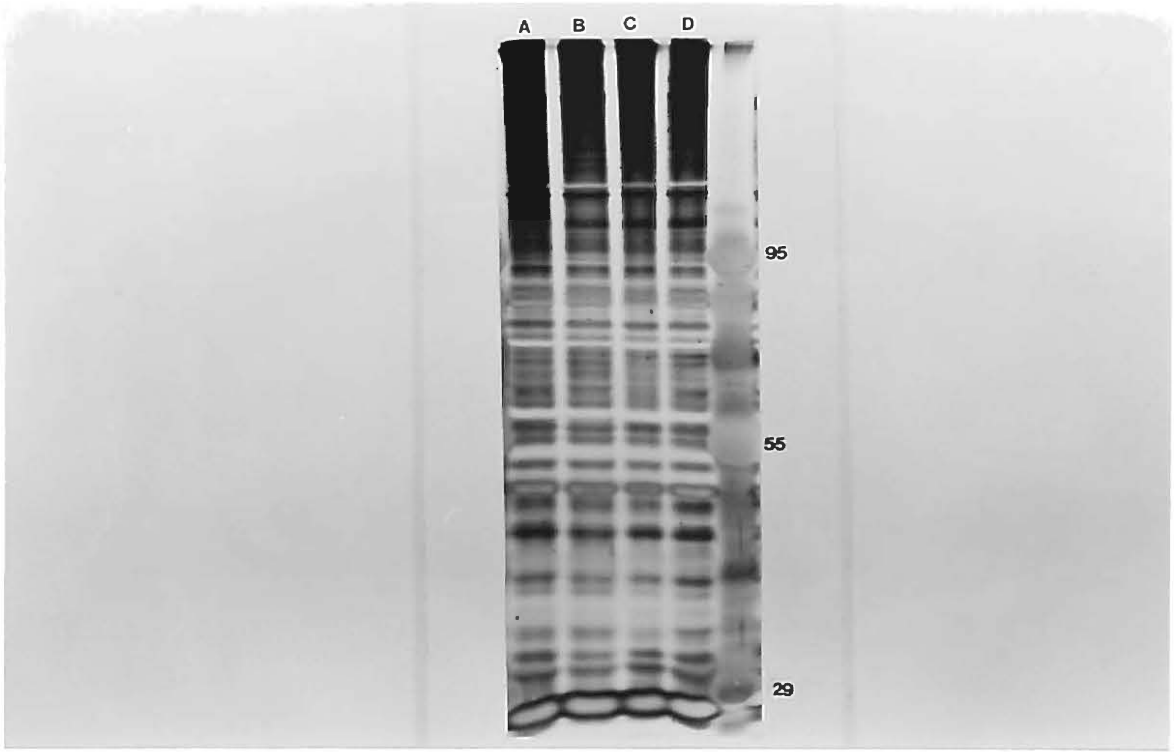

Fig. 6. Silver-stained SDS-PAGE gel of total protein profiles of S. citri cell cultures grown in either aerobic (lanes A,C) or anaerobic (lanes B,D) conditions. Numbers on the left represent the protein sizes of the standard in KDa.

CHAPTER IV

Protein Comparisons of *Spiroplasma citri* Lines Differing in Transmission or Subculturing History

ABSTRACT

Four lines of *Spiroplasma citri* Saglio strain BR3 differing in transmission or subculturing history were compared with respect to differences in 1) whole protein profiles, 2) production of four specific surface proteins, and 3) protein phase partitioning using Triton X-114. Proteins were assayed by one dimensional polyacrylamide gel electrophoresis and Western blotting. Although all four lines had similar profiles, each had one or more unique bands. Two of these, P29 and P89, were previously identified as having surface components (7,19). It is possible that one or more of the differences detected in the four experimental lines of *S. citri* is involved in activities related to pathogenicity or insect transmissibility.

INTRODUCTION

Phytopathogenic spiroplasmas, of the class Mollicutes, are transmitted by grafting, parasitic plants, and

arthropods (11). Proteins on the spiroplasma membrane surface may be involved in host-pathogen interactions such as adherence, enzyme actions, viral attachment, and pathogenicity, as demonstrated in several mycoplasma species (1,4,9,10,15,16,18). Approximately twenty-nine Spiroplasma citri membrane proteins have been identified (19,20,21). Twelve of these have surface epitopes, but their functions remain unknown (7).

Repeated passage of spiroplasmas in culture medium may result in the loss of either pathogenicity or transmissibility. Liu et al. (14) found that lines of S. citri MV101 subcultured more than five times and injected into Circulifer tenellus (Baker) were not transmitted. However, S. citri strain Palmyra was still transmitted to plants after 65 transfers in culture medium (12).

Four lines of S. citri differing in transmission and subculturing history were derived from strain BR3, which was originally isolated from Illinois horseradish plants with brittle root disease (5). BR3-T is a line maintained by leafhopper (C. tenellus) transmission in turnip plants in the greenhouse. BR3-G is a line that has been graft transmitted for eight years in periwinkle (causes symptoms on periwinkle but is no longer insect transmissible). BR3-M is a line resulting from 41 serial passages of BR3 in liquid medium (insect transmissibility and pathogenicity have not been tested). BR3-P has been subcultured in artificial

medium for 132 passages (plants exposed to leafhoppers microinjected with BR3-P did not become diseased). Recent experiments suggest that it is the ability to be transmitted, and not pathogenicity, that has been lost in BR3-P (Wayadande and Fletcher, personal communication). The purpose of this study was to compare the protein profiles of these four lines of S. citri BR3 to try to identify proteins which may play a role in the insect transmissibility and/or pathogenicity of spiroplasmas.

MATERIALS AND METHODS

Sample preparation. All experiments were carried out using four lines of Spiroplasma citri BR3-3x, which was originally isolated from Illinois horseradish affected with brittle root disease (5). The original isolate was triply cloned. BR3-T was freshly isolated from turnip, Brassica rapa, for this study, while BR3-G was freshly isolated from periwinkle, Catharanthus roseus. Both were increased in LD8 broth (13) at 31 C. BR3-M was subcultured 41 times in LD8, and BR3-P was subcultured 132 times in LD8. All lines were stored in small aliquots at -80 C, and individual tubes were thawed as needed.

Cell collection, Protein concentration and standardization, PAGE, and Western blotting The techniques were described in Chapter 3. For PAGE, the 10% gel was replicated 6 times, the 7.5% gel was replicated 3 times, and

the 5% gel was replicated 3 times. Western blots were developed with antiserum against whole S. citri cells, specific S. citri surface proteins (a mixture of anti-p89, anti-p77, anti-p58, and anti-p29 sera), or the surface protein P89. Each blot was replicated five times.

Triton X-114 phase partitioning. Following the modified version of Bordier (17), proteins of S. citri BR3 lines were separated into hydrophilic and hydrophobic fractions by Triton X-114 (TX-114) phase partitioning. TX-114 (Sigma Chemical Co., St. Louis, MO), prewashed and condensed, was added to a final concentration of 2% (w/v) to 1 ml cell culture (1 mg/ml, determined by the Bio-Rad protein assay) in N-2-hydroxyethyl-piperazine-N'-2-ethanesulfanic acid, pH 7.5, containing 10% sucrose (HEPES-S), and incubated 30 min at 4 C. Insoluble material was pelleted at 13000xg for 5 min at 4 C and the supernatant was transferred to a new tube, incubated for 5 min at 37 C, and centrifuged at 13000xg for 5 min at 4 C. The aqueous (upper) phase was removed to another tube and TX-114 was added to 2% again. HEPES-S was added to the detergent (lower) phase to bring it to original volume. The phase separation process was repeated for a total of six times.

RESULTS

PAGE and Western blotting. Significant differences were observed in the total protein profiles of the four

experimental lines of S. citri (Figures 1,2,3,4,5; Table 1). Although a majority of proteins were common to all lines, each line had one or more unique bands. Most protein differences among the lines were in bands between 84.1 and 175 KDa. Bands were given letter designations to reflect their relative positions in the profiles, rather than using calculated molecular masses, which varied slightly from gel to gel.

In PAGE experiments (Figures 1,2) bands q (89.1 KDa), a (88 KDa), and w'(127.5 KDa) were present in all four lines. Bands h (175 KDa), b (87.3 KDa), y (84.1 KDa), and possibly w (128.5 KDa), were present in BR3-G. Bands h, w, z' (91.4 KDa), and b were present in BR3-T. Bands z' and z (90.7 KDa) were present in BR3-M. Bands h, z, and b were present in BR3-P. Band i (39.7 KDa), missing in BR3-G, was present in the other three lines. Band s (27.5 KDa, spiralin) sometimes appeared as a doublet in BR3-G (s,s'(24 KDa)). Results of a Western blot developed using anti-spiralin serum confirmed that these two bands were serologically related. Table 1 summarizes the results and molecular weights of all gels.

Protein differences also were observed in Western blots incubated with antiserum to a whole cell preparation, a mixture of antisera specific for the four surface proteins P89, P77, P58, and P29, and antiserum against P89. In blots developed with antiserum specific for whole S. citri cells

(Figure 3) bands a (89 KDa) and c (86 KDa) were present in all four lines, though band c was less prevalent in BR3-P. Bands b (88 KDa), d (82 KDa), and g (59 KDa) were present in BR3-P while band y (78 KDa) was present in BR3-G. Band s (29 KDa) appeared in all four lines, though sometimes it was present as a doublet (s',26 KDa) in BR3-G.

In blots developed with a mixture of antisera specific for four surface proteins, band a appeared in all four lines, though it was most prevalent in BR3-G. Bands b (88 KDa), d (82 KDa), and g (59 KDa) were present in BR3-P while band y (78 KDa) was present in BR3-G. Band c (86 KDa) was present in all four lines though reduced in BR3-T and BR3-P. Band s (28 KDa) appeared in all four lines, though sometimes it was present as a doublet (s', 24 KDa) in BR3-G (Figure 4).

In blots developed in antiserum specific for P89, bands a (89 KDa) and c (86 KDa) appeared in all four lines, though band c was reduced in BR3-T and BR3-P. Bands b (88 KDa), d (82 KDa), and g (59 KDa) were present in BR3-P (Figure 5).

Triton-X phase partitioning. When treated with whole cell antiserum (Figure 6), bands corresponding to the proteins of the four lines appeared in both phases. Bands were given letter designations to relect their relative positions in the profiles (not related to previous letter designations), rather than using calculated molecular masses, which varied slightly from blot to blot. Two

adjacent bands of approximately 77 KDa separated into different phases, with the upper band (c) appearing in the detergent phase and the lower band (d) in the aqueous phase. A 58 KDa band (f) partitioned into the aqueous phase. The 29 KDa band (g) appeared most prominently in the detergent phase. A protein of 127 KDa (a) appeared in BR3-M in the detergent phase only. In BR3-P, a band of 59 KDa (e) was visible in the detergent treatment. It was not possible to distinguish clear separation of protein bands in the P89 region (b) in blots developed with either antiserum. In blots developed with anti-P89 serum (Figure 7) a band of 127 KDa (a) appeared in the detergent phase in BR3-M, and a band of 59 KDa (c) was visible in the detergent phase in BR3-P. Similar banding patterns occurred when the experiment was repeated.

DISCUSSION

The S. citri lines differing in transmission or subculturing history appear to have several protein differences. PAGE revealed two bands, z′ and possibly w, that appeared in the transmissible line BR3-T but were not present in the non-transmissible lines BR3-G and BR3-P. Other bands appeared in BR3-G (y,s′) and BR3-P (z) that are not present in the transmissible line. The protein profile of BR3-M, the line in which transmissibility and pathogenicity have yet to be tested, contained some

similarities to the transmissible line (z'), some similarities to non-transmissible lines, and one feature unique to BR3-M (lack of band b). In Western blots developed with antiserum specific for S. citri whole cells and a mixture of antisera specific for P89, P77, P58, P29, additional proteins appeared in the nontransmissible lines BR3-G (y,s') and BR3-P (b,d,g) not seen in BR3-T and BR3-M along with a band, c, which was reduced in BR3-T and BR3-P. In blots developed with antiserum specific for P89, additional bands were present in BR3-P (b,d,g) not present in BR3-T and the other lines, and band c was very much reduced in BR3-T and BR3-P. Bands d and g probably represent degradation products of the 89 KDa group, since they react with anti-P89 serum.

It could be that the proteins present in BR3-G and/or BR3-P but missing in BR3-T interfere with insect transmissibility. They may block recognition of the spiroplasmas within the insect. Lack of a certain protein may also hinder recognition, if that protein is required for the interaction. BR3-T has at least one, and possibly two, unique proteins not seen in the non-transmissible lines. However, whether these protein differences are actually related to transmissibility is unclear. BR3-P grows more quickly in liquid medium than do the other three lines (unpublished data). Possibly, the unique proteins observed in this line are involved with this phenomenon. The fact

that BR3-P and BR3-G, both non-transmissible by insects, do not have identical protein profiles is not surprising. These strains have come about because of natural mutation and may have lost transmissibility as a result of different mutations. The protein pattern of BR3-M may provide a key to which protein(s) may be involved in transmissibility once its own transmissibility phenotype has been determined.

A notable protein difference was the mobility of the protein spiralin in BR3-G compared to that in the other lines. This protein, in line BR3-G only, appeared sometimes as a band at approximately 29 KDa, sometimes as a band at approximately 26 KDa, and occasionally as a doublet. Proteins of both mobilities reacted in Western blots with anti-p29 serum. Variations in the electrophoretic mobility of spiralin have been observed in several S. citri strains (8). The mechanism inducing this polymorphism is not known. Saillard and colleagues (8) have demonstrated that the variations are not caused by size differences of N-terminus modification of the spiralin amino acid sequence. They suggest that acylation may alter the mobility of spiralin.

Several adjacent protein bands appeared in Western blots of the four lines when blots were developed with p89 antiserum. These may be variants, derivatives, or breakdown products of the 89 KDa protein to which the antiserum was developed. Or, these bands may be individual proteins of similar mass that were inadvertently excised together from

preparative gels and injected together into the rabbits during antiserum development.

To determine whether these adjacent proteins were different proteins with close molecular mass, proteins of the different lines were fractionated into aqueous and detergent phases by Triton X-114 phase separation and visualized by Western blotting. Generally, proteins fractionating in the aqueous phase are cytoplasmic proteins, while those in the detergent phase are likely to be proteins on/in the membrane of an organism. If certain bands appeared in one phase, while other bands appeared in the other phase, the bands probably represent different proteins. Previous work (6) showed that P77 often occurs as a doublet with the upper band partitioning into the hydrophobic phase and the lower band into the hydrophilic phase; P58 is hydrophilic, separating into the aqueous phase; and P29 is a hydrophobic protein, partitioning into the detergent phase (6). Similar partitioning was seen for P77 and P58 in the blots in this study. The 29 KDa band appeared most prominently in the detergent phase. The appearance of a light band at 29 KDa in the aqueous phase is attributed to well overflow, since it also occurs in the standard lane and in lanes not containing any sample.

Previously, P89 was demonstrated to be hydrophobic, paritioning into the detergent phase (6). In this study bands appeared in the p89 region in both phases. Because of

poor band resolution in the detergent phase, it was impossible to determine whether bands of the same molecular mass appeared in both phases. This could occur if the proteins were not completely fractionated into aqueous/detergent phases; however, after six cycles of fractionation P77 and P58 had partitioned completely into their respective phases. Previous experiments have shown clear separation of the phases after four fractionation cycles (6). A second possibility is that several proteins with similar molecular mass are present. Finally, it could be that one or more of the proteins of this approximate molecular mass (89 KDa) contain approximately equal hydrophobic and hydrophilic regions. If this were the case, some of the protein molecules might tend to fractionate in each of the two phases.

Zoopathogenic mycoplasma species such as M. pneumoniae have specific surface proteins which function in recognition of host cells, attachment, and enzymatic activities (1,4,9,10,15,16,18). It is possible that one or more of the proteins which are differentially expressed in the four experimental lines of S. citri is involved in similar activities related to pathogenicity or insect transmissibility. Further characterization of such proteins may contribute to our knowledge of the determinants of these critical events.

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TABLE I. Polyacrylamide gel summary of total proteins of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G, BR3-T, BR3-M, AND BR3-P

BR3-G	BR3-T	BR3-M	BR3-P	Approx MW
h	h		h	175
w[a]	w			128.5
w′	w′[b]	w′	w′	127.5
	z′	z′		91.4
		z	z	90.7
q	q	q	q	89.1
a	a	a	a	88
b	b		b	87.3
y				84.1
	i	i	i	39.7
s	s	s	s	27.5
s′[c]				24-26

[a] w possibly present in 5% gels, not seen in 7.5% gels.
[b] w′ present in 5% gels, not seen in 7.5% gels.
[c] Detection of band s′ in BR3-G was inconsistent.

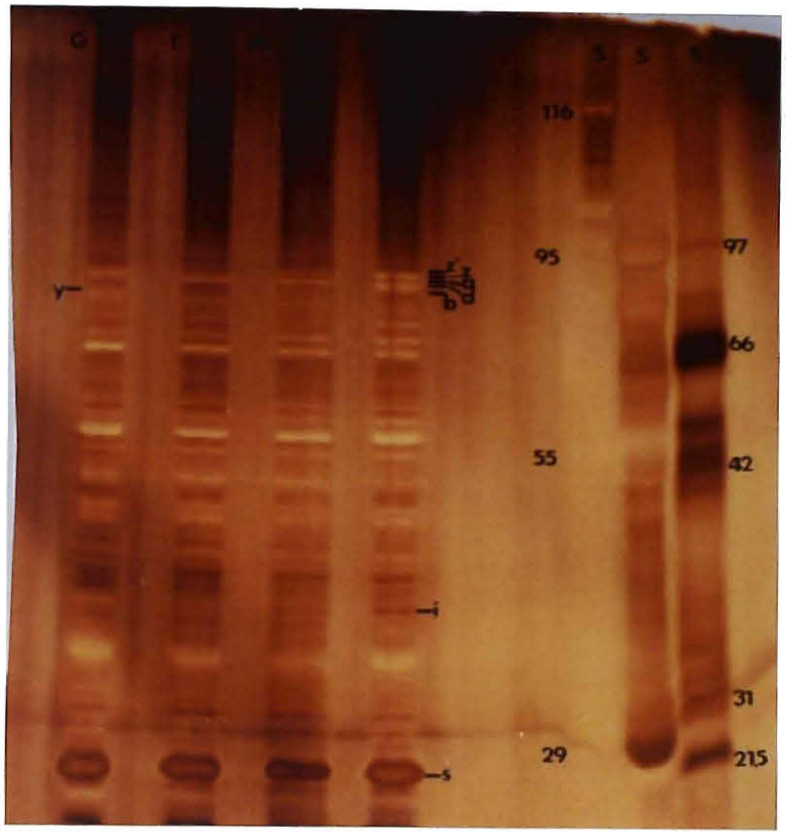

Fig. 1. Silver-stained 10% SDS-PAGE gel of total protein profiles of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (lane G), BR3-T (lane T), BR3-M (lane M), and BR3-P (lane P). Lanes S (molecular weight standards). The numbers on the left and right of the standard lanes represent protein sizes of the standards in KDa. Letters a, b, i, q, s, y, z, and z' represent differences in the protein banding patterns in the four lines.

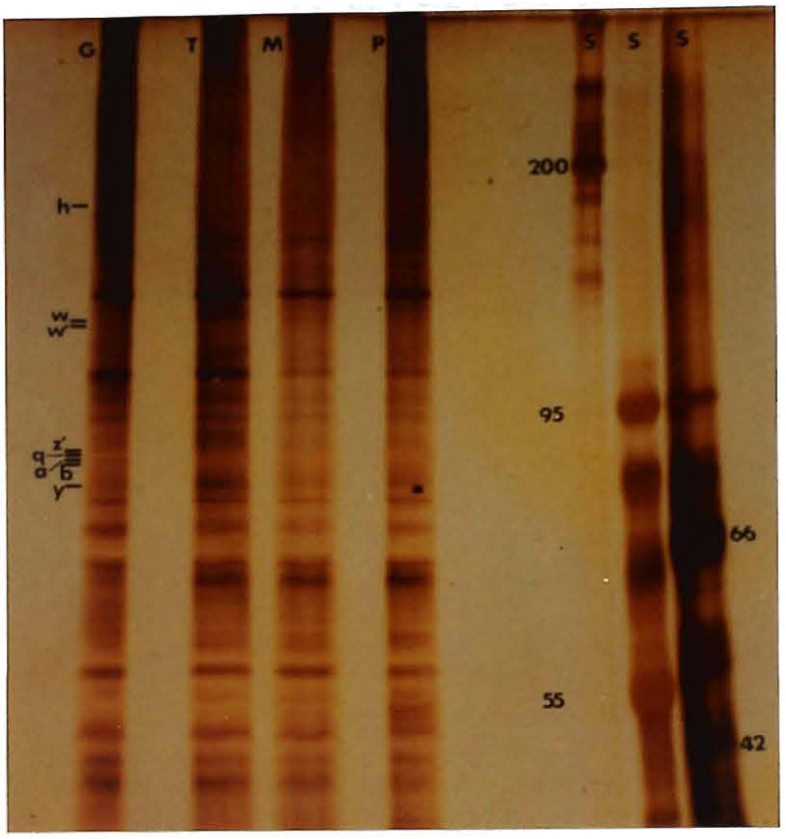

Fig. 2. Silver-stained 7.5% SDS-PAGE gel of total protein profiles of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (lane G), BR3-T (lane T), BR3-M (lane M), and BR3-P (lane P). Lanes S (molecular weight standards). Numbers to the right and left of the standard lanes represent protein sizes of the standards in KDa. Letters a, b, h, q, w, w', y, z' represent differences in the protein banding patterns in the four lines.

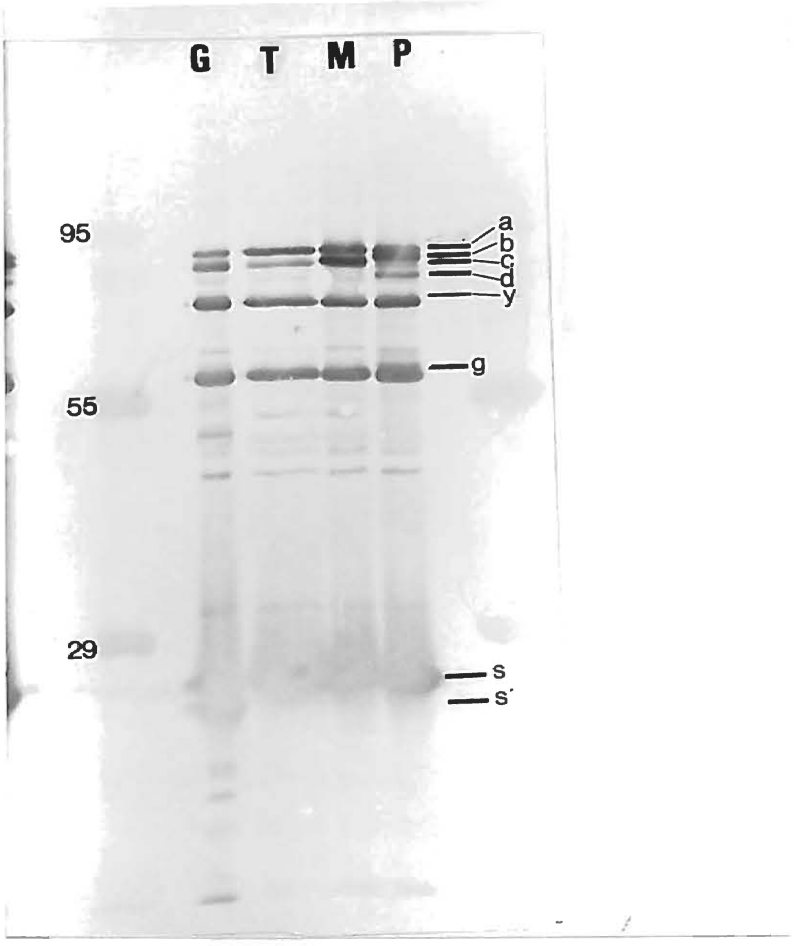

Fig. 3. Western blot of total protein profiles of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (lane G), BR3-T (lane T), BR3-M (lane M), and BR3-P (lane P). Blot developed with antiserum specific for a whole cell preparation of S. citri. Numbers to the right represent proteins sizes of the standard in KDa. Letters a, b, c, d, g, s, s' and y represent differences in the protein banding patterns in the four lines.

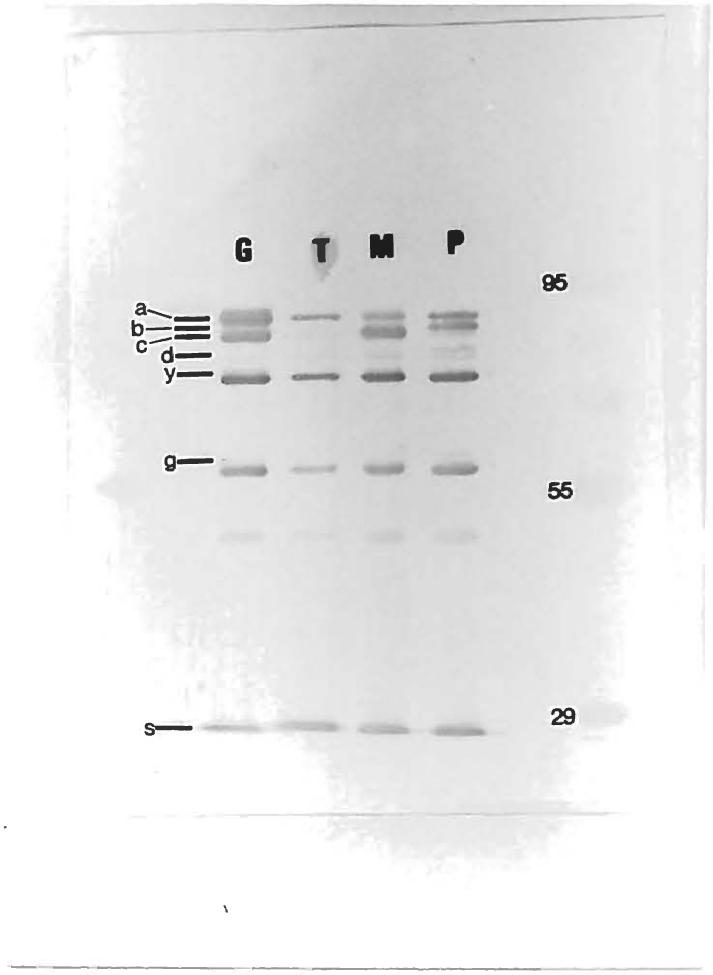

Fig. 4. Western blot of total protein profiles of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (lane G), BR3-T (lane T), BR3-M (lane M), and BR3-P (lane P). Blot developed with a mixture of antisera specific for p89, p77, p58, and p29. Numbers on the right represent protein sizes of the standard in KDa. Letters a, b, c, d, g, s, and y represent differences in the protein banding patterns in the four lines.

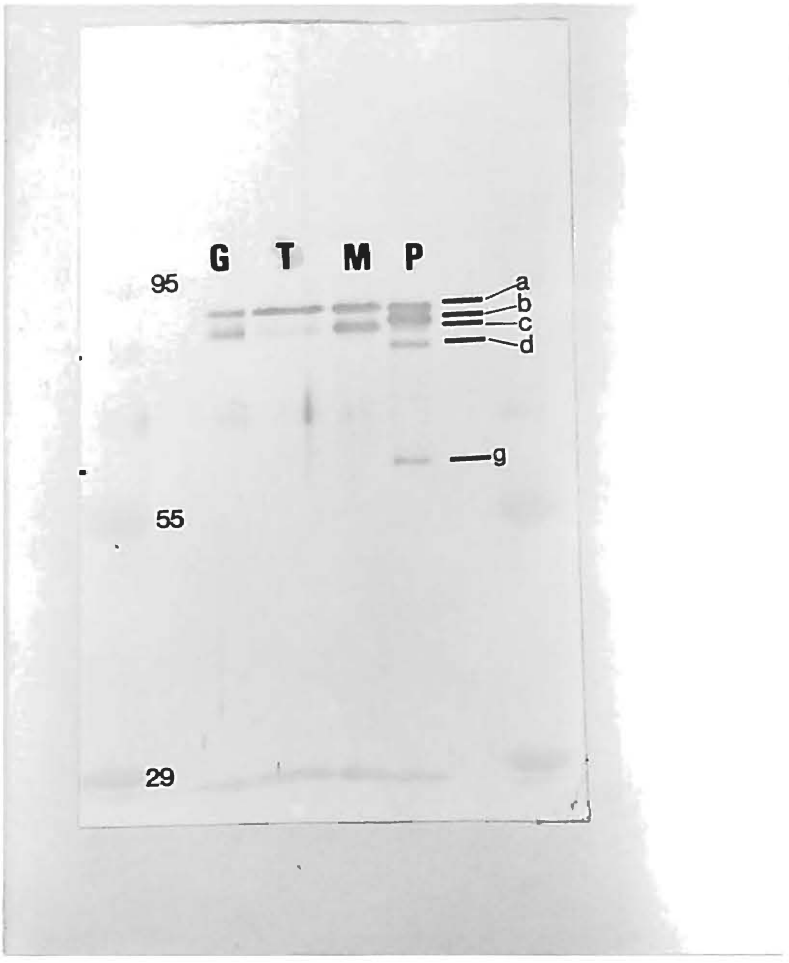

Fig. 5. Western blot of total protein profiles of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (lane G), BR3-T (lane T), BR3-M (lane M) and BR3-P (lane P). Blot developed with antiserum against p89. Numbers appearing on the left represent protein sizes of the standard in KDa. Letters a, b, c, d, and g represent differences in the protein banding patterns in the four lines.

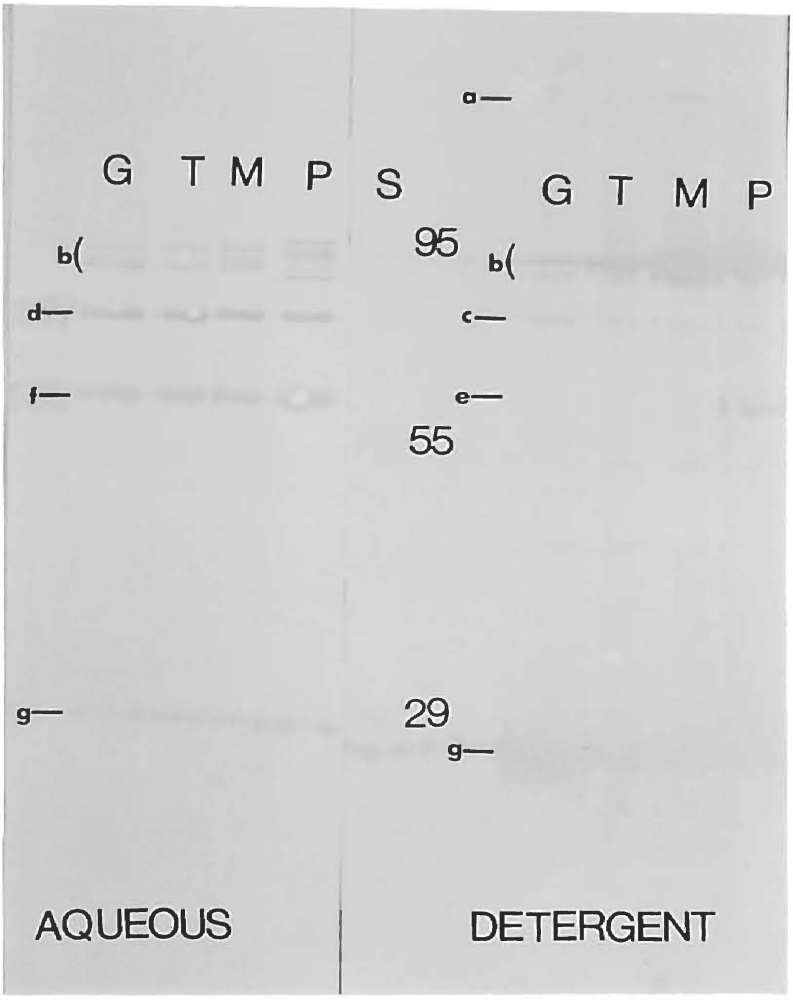

Fig. 6. Western blot of Triton-X phase partitioned total proteins of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (G), BR3-T (T), BR3-M (M), and BR3-P (P). Blot developed with antiserum specific for whole S. citri cells. Lanes S (molecular weight standard). Numbers appearing in the middle represent protein sizes of the standard in KDa. Letters a, b, c, d, e, f, and g represent proteins of the four lines.

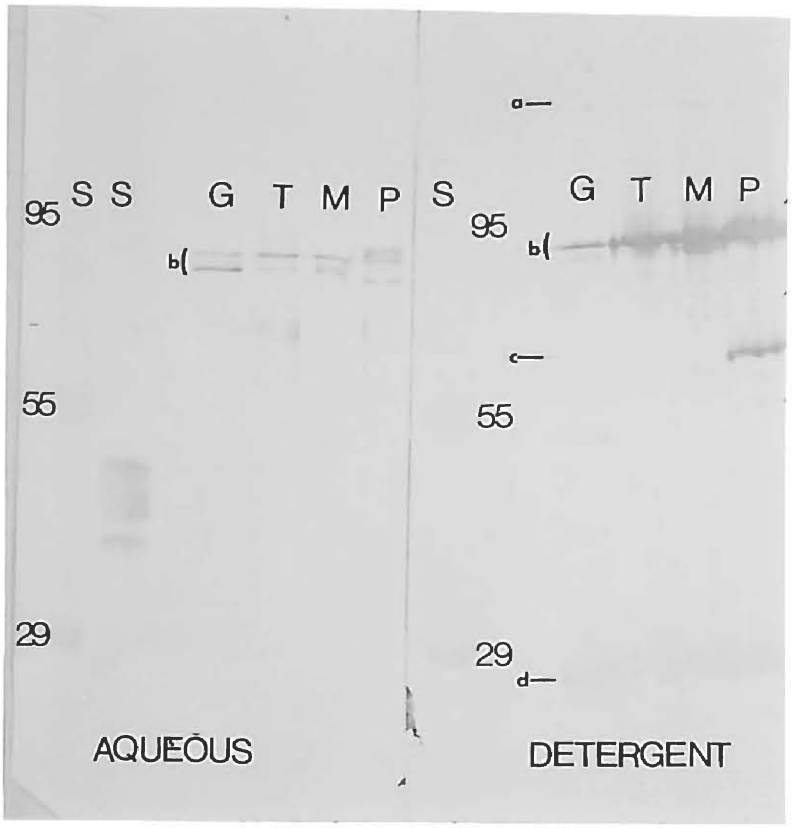

Fig. 7. Western blot of Triton-X phase partitioned total proteins of four lines of S. citri strain BR3 differing in transmission or subculturing history: BR3-G (G), BR3-T (T), BR3-M (M), and BR3-P (P). Blot developed with antiserum against p89. Lanes S (molecular weight standard). Numbers represent protein sizes of the standard in KDa. Letters a, b, c, and d represent proteins of the four lines.

CHAPTER V

Comparisons of the Protein Profiles and Colony Morphology of the Non-Helical *Spiroplasma citri* strain ASP-1 with Helical Strains

ABSTRACT

Spiroplasma citri strain ASP-1 is an unusual spiroplasma in that it was reported to be both non-helical and non-motile, but pathogenic (9). However, recent pathogenicity tests with ASP-1 have been negative (C.J. Chang, personal communication). Previous work by Townsend (10) indicates that ASP-1 lacks a single protein of 39 KDa when compared with motile strains. This protein may play a part in helicity and motility of spiroplasmas. The objective of this project was to compare ASP-1 to helical *S. citri* strains with respect to 1) total protein profiles, 2) the presence of four specific surface proteins, and 3) colony motility and morphology on agar. Proteins were assayed by one dimensional polyacrylamide gel electrophoresis and Western blotting. Silver-stained SDS-PAGE gels and Western blots indicated more protein differences between ASP-1 and the helical strains than were originally reported. The ASP-1 strain was non-motile and

produced well-defined "fried egg" colonies without satellites.

INTRODUCTION

Spiroplasmas, members of the class Mollicutes, are wall-less pleomorphic prokaryotes (12). The term spiroplasma was coined in 1973 to reflect the helical morphology of one subgroup of Mollicutes (1). Motility and morphology are two major features which distinguish most spiroplasmas from other Mollicutes (10). However, Spiroplasma citri strain ASP-1 is non-motile and non-helical (9).

Although it lacks motility and helicity, ASP-1 shares many other spiroplasma characteristics (9,10). It is infected by three spiroplasma-associated viruses, SV-C1, SV-C2, and SV-C3. Symptoms produced in plants by this strain are identical to those in plants infected by helical S. citri strains. Optimal growth temperature in vitro is 32 C.

In liquid medium, ASP-1 cells tend to form aggregates and the lag phase is longer than that of helical strains. On agar-solidified media, ASP-1 produces transparent, smooth, well-defined "fried egg" colonies without satellites, while helical strains produce diffuse "fried egg" colonies with satellites. ASP-1 lacks a 39 KDa protein present in helical S. citri strains (9,10).

A protein missing in ASP-1 but present in helical

strains could be involved with helicity and/or motility of spiroplasmas. The purpose of this study was to compare the electrophoretic protein profiles, morphology, and motility of the non-helical S. citri strain ASP-1 with those of helical S. citri strains.

MATERIALS AND METHODS

Cell preparation. Spiroplasma citri strain BR3 was originally isolated from horseradish plants with brittle root disease in Illinois (3). BR3-29 is a line of BR3 serially passed 29 times in liquid medium and BR3-G is a line that has been graft transmitted for eight years in periwinkle. S. citri strains ASP-1 and R8A2 were obtained from R. E. Davis, USDA, Beltsville. Flasks containing LD8 medium (7) were inoculated with all the strains at an initial titer of $2x10^5$ cells/ml. Cultures were incubated at 32 C.

Cell collection, Protein concentration standardization, PAGE, and Western blotting. The procedures were described in Chapter 3.

Plating. Serial dilutions of ASP-1 and BR3-M (100 ul) were plated on agar-solidified LD8 medium (7). Cultures were incubated at 32 C. After four days, 3 drops of Dienes' stain (2) were added to each plate for 30 sec and washed off with distilled water. Colonies were observed with a dissecting microscope.

RESULTS

PAGE. Differences were observed when the protein profiles of S. citri strain ASP-1 were compared to those of the helical strains (Figures 1,2). ASP-1 lacked a band of 38.1 KDa present in the helical strains. A 26 KDa protein band consistently present in R8A2 and BR3-28 was missing in ASP-1, but a 24 KDa protein was present. A 46.2 KDa protein band present in BR3-29 was missing in R8A2 and ASP-1.

Western blots. Blots of ASP-1, R8A2, and BR3-29 were developed with antiserum against S. citri whole cells (Figure 3). ASP-1 and BR3-29 shared similar banding patterns, except that a 21.3 KDa band present in ASP-1 was not found in BR3-29, while a band of 24.9 KDa present in BR3-29 (and R8A2) did not occur in ASP-1. R8A2 had bands of 93.7 KDa, 89.9 KDa, 85.2 KDa, and 31.3 KDa not seen in ASP-1 or BR3-29.

Blots of ASP-1, R8A2, and BR3-29 were also developed with a mixture of antisera against four surface proteins, p89, p77, p58, and p29 (4) (Figure 4). The banding patterns were virtually the same as with the whole cell antiserum, except that the band of approximately 84.3 KDa was visible in ASP-1 and BR3-29, as well as in R8A2. This band was still more prevalent in R8A2, however.

Blots of ASP-1 and BR3-G were developed with antiserum specific for S. citri whole cells (Figure 5). ASP-1 lacked

two bands (89 KDa, 87 KDa) present in BR3-G, and a band of 92 KDa appeared to be much less prevalent in ASP-1 (not visible) than in BR3-G. A 54 KDa band appeared more prevalent in BR3-G, while a 48 KDa band appeared more prevalent in ASP-1. Two bands (27.5 KDa and 26 KDa) in BR3-G are missing in ASP-1, but a band of 23 KDa occurred in ASP-1. In Western blots developed with anti-spiralin serum, the 27.5, 26, and 23 KDa bands all appeared, suggesting that they are different forms of spiralin (data not shown).

A blot of ASP-1 and BR3-G developed with a mixture of antisera against four surface proteins, p89, p77, p58, and p29 (4), showed similar banding patterns except for the absence of the 48 KDa and 54 KDa bands (Figure 6).

Plating. The ASP-1 culture exhibited well-defined "fried egg" colonies without satellites (indicative of non-motile cells, Figure 7) while S. citri strain BR3-M colonies were diffuse and contained satellites (indicative of motile cells which travel through the agar, Figure 8).

DISCUSSION

Previous work by Townsend (10) indicated that the nonhelical S. citri strain ASP-1 lacks a single protein, 39 KDa, when compared with motile strains. He suggested that this protein plays a part in helicity and motility of spiroplasmas. This research, in which a culture of ASP-1 obtained from R. E. Davis, USDA, Beltsville, was compared to

S. citri strain R8A2 (the strain to which it had been compared previously (10)) and to a horseradish brittle root isolate of S. citri, BR3, confirmed the absence of a 39 KDa protein in ASP-1 previously reported by Townsend et al. (9,10). However, in this study there was also a significant difference in the PAGE mobility of spiralin in ASP-1 compared to that in helical strains (seen in Western blots developed with anti-spiralin serum).

Variations in the electrophoretic mobility of spiralin have been observed previously, by others, in several S. citri strains. Foissac and colleagues (5) demonstrated that the spiralin variations seen in strains C189, R8A2B, Corse, ASP-1, Israel, Alcanar 254, Alcanar 78, and Palmyre are not caused by size differences due to N terminus modification of the spiralin amino acid sequence. They suggest that acylation may alter the mobility of spiralin.

Spiralin sometimes appears as a doublet in BR3-G (Chapter IV, this thesis). However, the spiralin band in ASP-1 had a greater mobility than the lower band of BR3-G. It is unlikely that these changes in spiralin are the cause of the loss of helicity since helical strains also exhibit altered forms of spiralin. The spiralin banding differences could be explained in several ways. S. citri strains may have a high mutation rate (11), which could contribute to the protein variations in the different spiroplasma strains (see Chapter IV). Alternatively, proteins in the different

strains might have undergone unequal degradation during sample preparation.

Western blots incubated in antiserum to S. citri whole cells or to a mixture of antisera to four specific surface proteins (P89, P77, P58, P29) have shown that P89 is less prevalent in ASP-1 than in the helical strains. It is unlikely that this reduction in P89 had an effect on helicity, since it was not prevalent in the helical strain BR3-29 either. Previous work (4) also showed a loss of P89 in S. citri R8A2, S. melliferum AS576, and S. kunkelii CSS, other helical strains. Why P89 did not appear in ASP-1 and BR3-29 when blots were incubated with antiserum to S. citri whole cells is not known. Since both P77 and P58 were present in all strains it is unlikely that they have an effect on helicity.

Townsend (9) reported that the non-helical ASP-1 produces very distinct "fried egg" colonies, with no satellite colonies, when plated on agar, while helical strains produced diffuse colonies with satellites. The ASP-1 culture obtained from Davis exhibited non-motile, non-helical cells and well-defined "fried egg" colonies with no satellites.

Spiroplasmas are differentiated from other Mollicutes in part by their helicity and motility (6). ASP-1 was the first spiroplasma known to be both non-helical and nonmotile (8,9). More recently, non-helical and partially helical

mutants of Spiroplasma kunkelii have been described (8). In this species, a 38 KDa protein was found to be most prevalent in the helical strains, less prevalent in the partially helical mutants, and missing or present in minute amounts in the non-helical mutants. It is possible that this protein homologous to the 39 KDa protein which was absent in ASP-1 in this work and Townsend's (10). If so, the correlation of the 38 KDa protein with varying degrees of helicity in S. kunkelii supports the suggestion of Townsend that this protein could play a role in helicity. Thus, it appears that classifying an organism as a spiroplasma only by its helicity and motility may not be totally appropriate.

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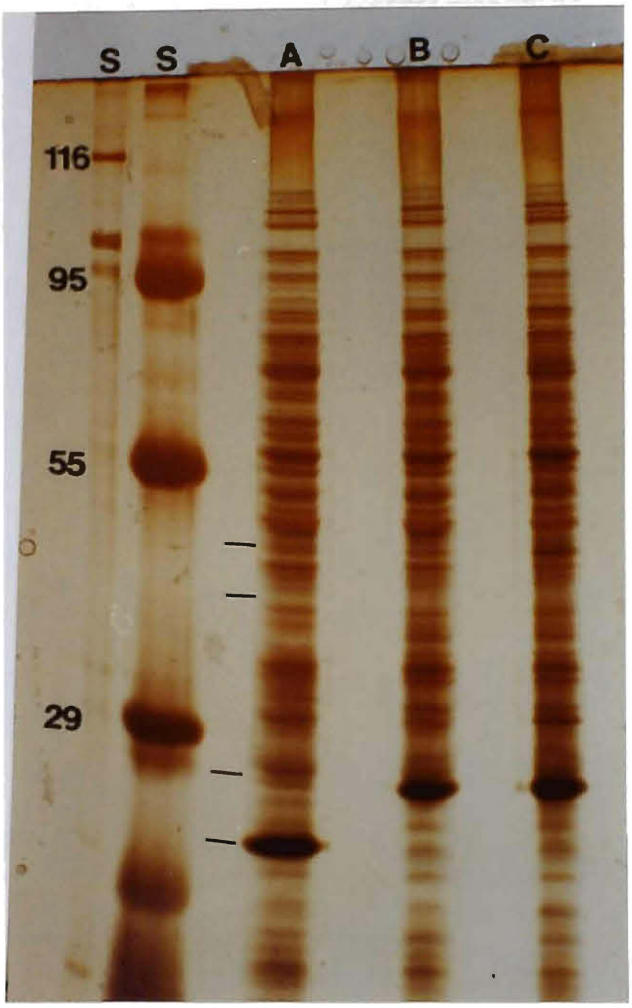

Fig. 1. Silver-stained 10% SDS-PAGE gel of total protein profiles of S. citri strain ASP-1 (lane A), S. citri strain R8A2 (lane B), and S. citri strain BR3-29 (lane C). Molecular mass marker (lanes S). Numbers to the right represent protein sizes of the standards in KDa. Bars represent differences in the protein banding patterns in the three lines.

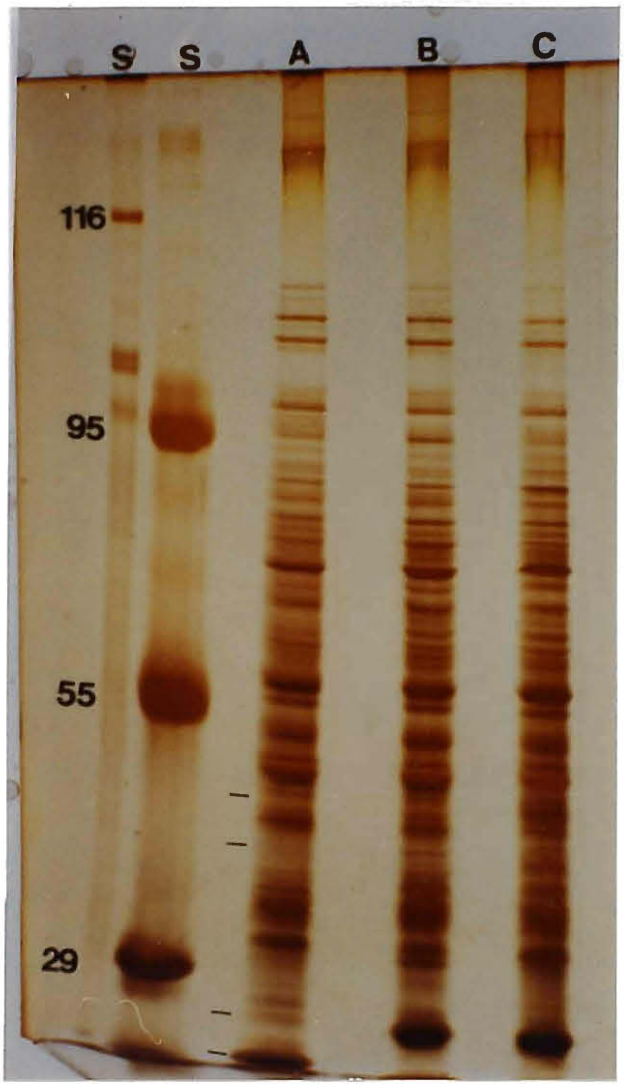

Fig. 2. Silver-stained 7.5% SDS-PAGE gel of total protein profiles of S. citri strain ASP-1 (lane A), S. citri strain R8A2 (lane B), and S. citri strain BR3-29 (lane C). Molecular mass marker (lanes S). Numbers to the right represent protein sizes of the standard in KDa. Bars represent differences in the protein banding patterns in the three lines.

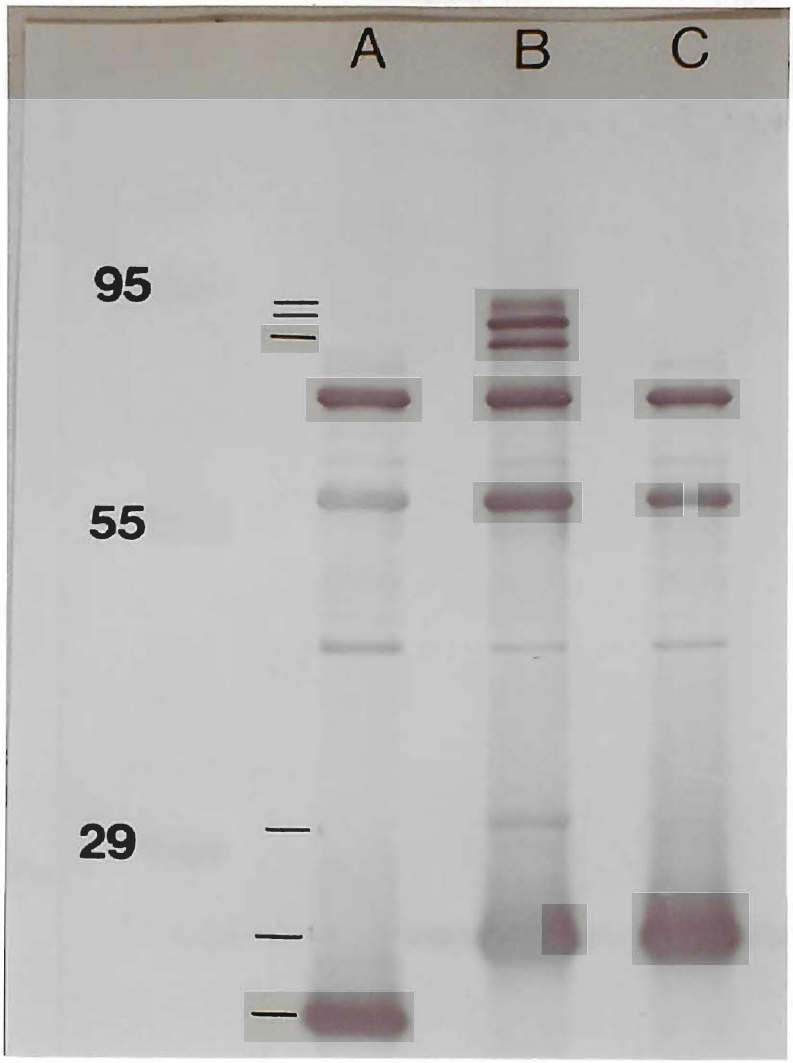

Fig. 3. Western blot of total protein profiles of S. citri ASP-1 (lane A), S. citri R8A2 (lane B), and S. citri strain BR3-29 (lane C). Blot developed with antiserum against whole S. citri cells. Numbers to the left represent proteins sizes of the standard in KDa. Bars represent differences in protein banding patterns in the three lines.

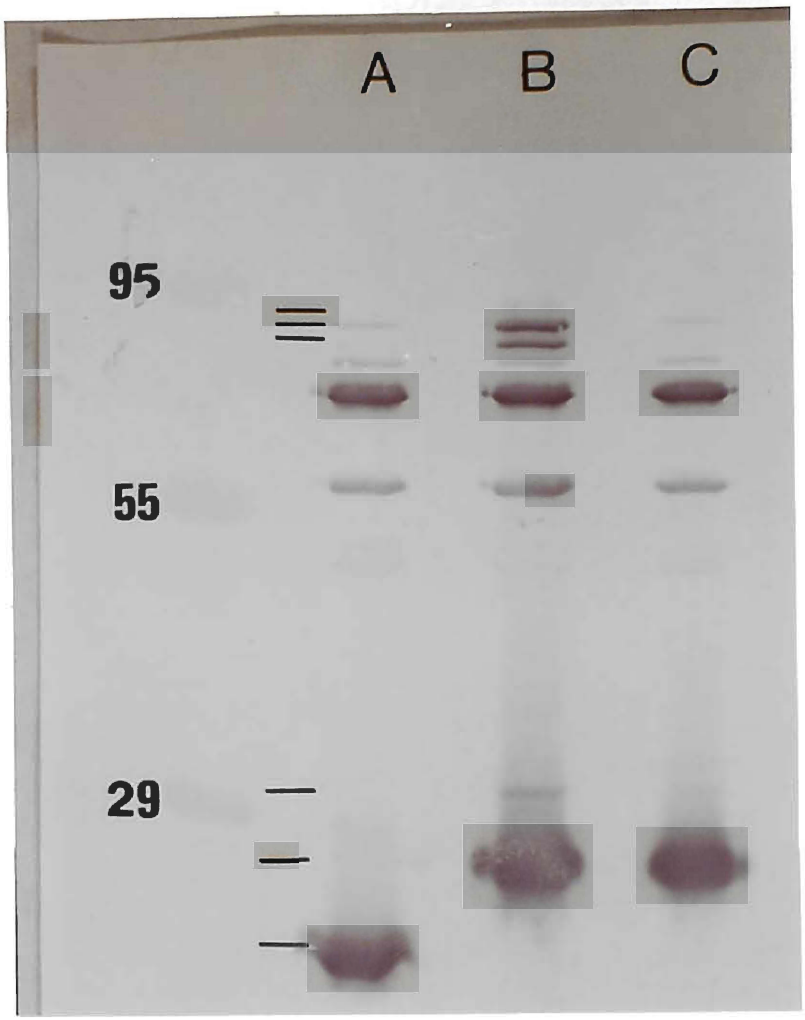

Fig. 4. Western blot of total protein profiles of S. citri ASP-1 (lane A), S. citri R8A2 (lane B), and S. citri BR3-29 (lane C). Blot developed with a mixture of p89, p78, p55, and p29 antisera. Numbers to the left represent protein sizes of the standard in KDa. Bars represent protein banding differences in the three lines.

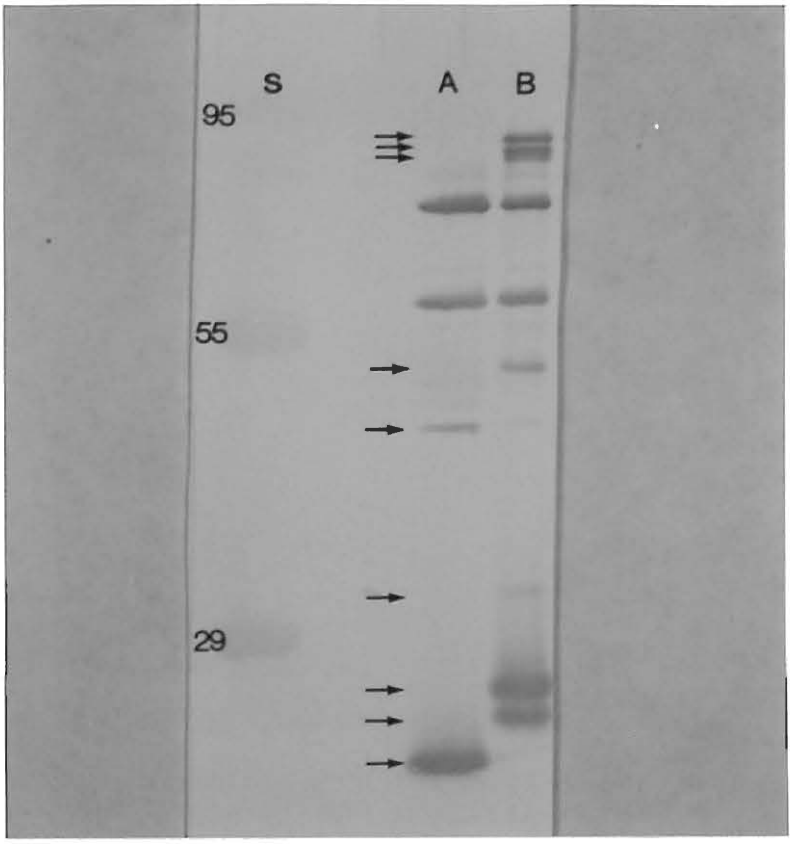

Fig. 5. Western blot of total protein profiles of S. citri strain ASP-1 (lane A) and S. citri strain BR3-G (lane B). Blot developed with antiserum against whole S. citri cells. Molecular mass marker (lane S). Numbers to the left represent protein sizes of the standard in KDa. Arrows represent protein banding differences in the two lines.

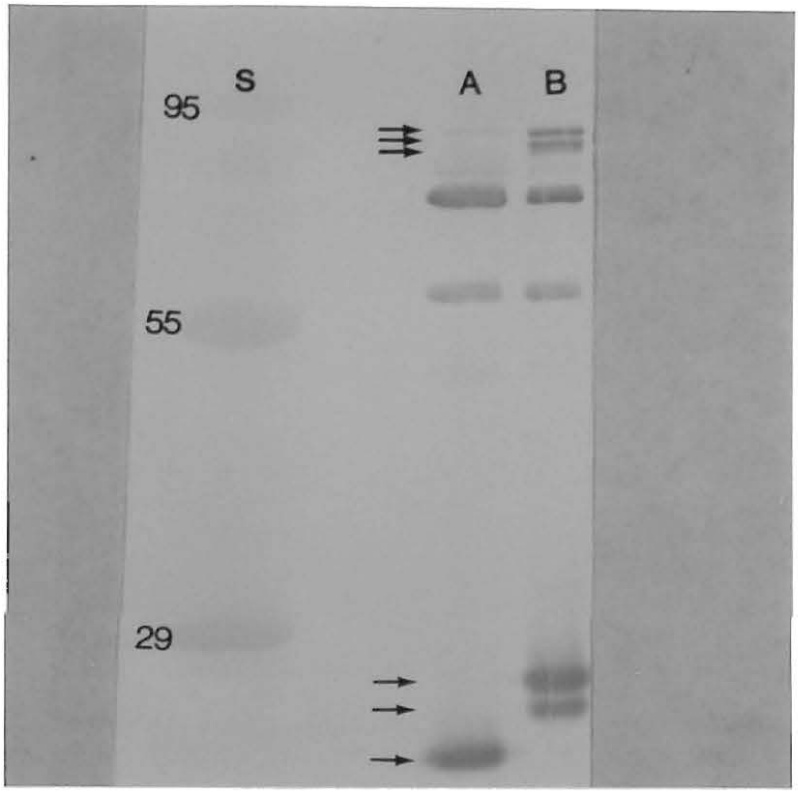

Fig. 6. Western blot of total protein profiles of S. citri strain ASP-1 (lane A) and S. citri strain BR3-G (lane B). Blot treated with a mixture of p89, p78, p55, p29 antisera. Molecular mass marker (lane S). Numbers to the left represent protein sizes of the standard in KDa. Arrows represent protein banding differences in the two lines.

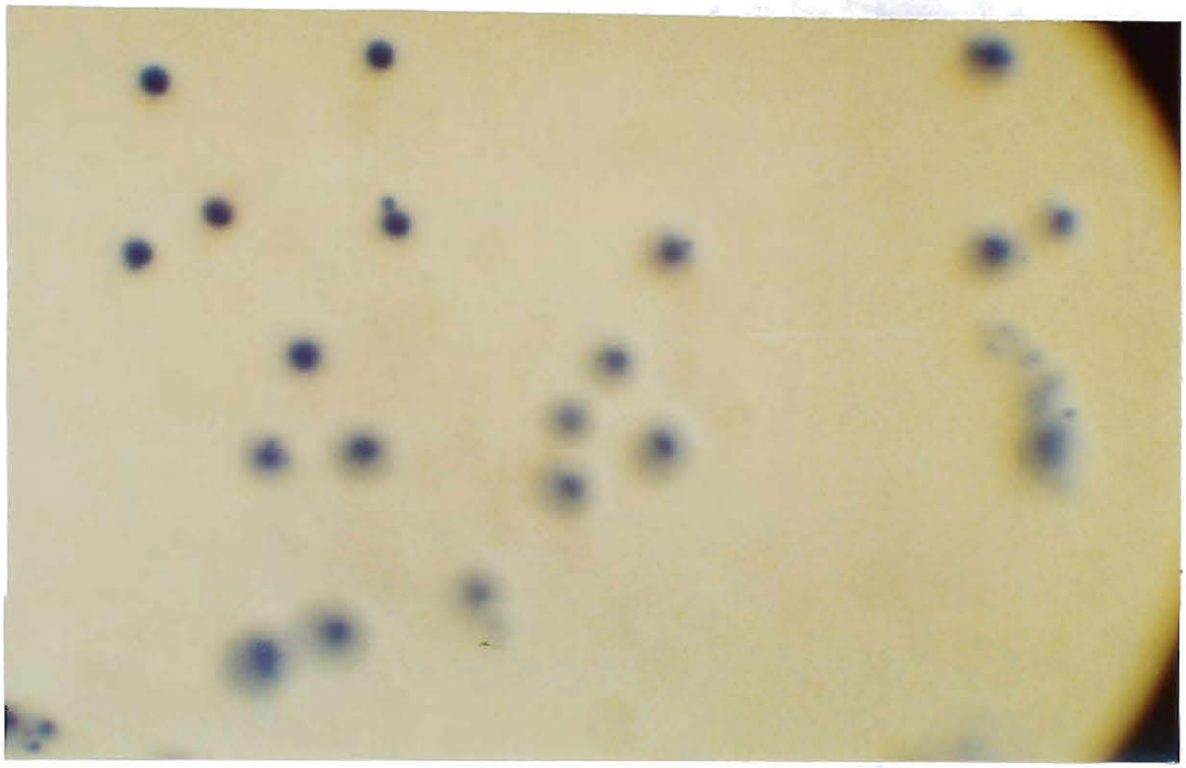

Fig. 7. Smooth, well-defined "fried egg" colonies of S. citri strain ASP-1 lacking satellites. Stained with Dienes' stain. Mag. 700x

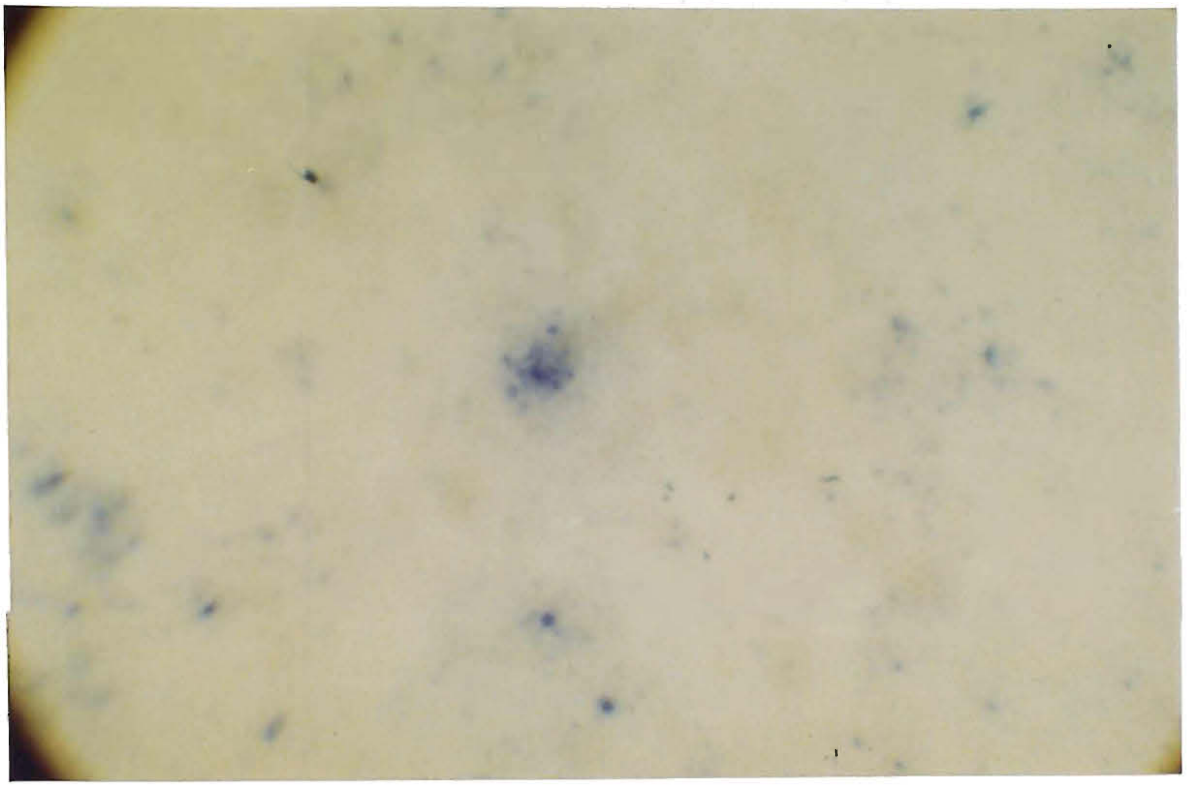

Fig. 8. Diffuse "fried egg" colonies of S. citri strain BR3-M with satellites. Stained with Dienes' stain. Mag. 700x

CHAPTER VI

MORPHOLOGY OF THE MIDGUT OF THE LEAFHOPPER CIRCULIFER TENELLUS

ABSTRACT

The meso-midgut of the beet leafhopper, Circulifer tenellus, is characterized by a layer of epithelium lining the lumen, with cells varying in shape and protruding into the lumen to varying depths. The filter chamber, or anterior midgut, is morphologically larger than the meso-midgut, but is very similar in ultrastructure. A basement membrane separates the midgut layers from the hemocoel. The epithelial cells are lined with microvilli which extend into the lumen. Moving outward from this layer are a second layer of epithelial cells and a layer of muscles. Cells are sometimes binucleate, and contain endoplasmic reticulum, ribosomes, and vacuoles. Numerous mitochondria are associated with the muscle cells and microvilli. The leafhopper harbors as yet unidentified bacteria-like organisms in the meso-midgut and anterior midgut lumens and in membrane-bound pockets in epithelial cells.

INTRODUCTION

The major vector of Spiroplasma citri is the beet

leafhopper, Circulifer tenellus (Baker). The life cycle of this insect usually includes two to three generations per year. Females are fertilized in the fall and the adult leafhoppers overwinter on weed hosts. In late winter and early spring 300-400 eggs per female are deposited on plant leaves and stems, where hatching is temperature-dependent. As soon as the nymphs hatch, they can feed and aquire spiroplasmas. Maturation is complete in 3-6 weeks, after five molts (3).

The digestive tract of C. tenellus has not been decribed in detail, but Liu et al. (5) examined the leafhopper gut in their study of the interactions of S. citri with C. tenellus. The internal anatomy of other members of the leafhopper family, Cicadellidae, has been described, including Euscelis spp., Graminella nigrifrons (Forbes), and Euscelidius variegatus (Kirschbaum) (1,6). Collectively, the alimentary canal of the Cicadellidae is composed of the maxillary stylets, cibarium, esophagus, and gut (foregut, midgut, and hindgut) (Figure 1). Fluid is sucked up through the stylets by cibarial pump action. The fluid then passes through the cibarium into the esophagus, which opens into the midgut. A filter chamber, located within the anterior midgut, shunts water entering from the foregut directly to the hindgut, thus concentrating the nutrients in the midgut. A suspensory ligament joins the midgut and filter chamber to the esophagus. Malpighian

tubules are joined to the posterior end of the midgut, allowing waste products to enter the hindgut (1,6).

The midgut is endodermal in origin. The lumen is bounded by a layer of epithelial cells, normally binucleate and varying in shape. These cells extend into the lumen in a row of closely packed, double or multimembraned microvilli which collectively are referred to as a striated brush border. A muscle layer, containing different muscle cell types, surrounds the epithelial cells. Outside this muscle layer is a basement membrane, which separates the midgut from the hemocoel. Rough endoplasmic reticulum, scattered mitochondria, and large lysosome bodies are present inside the epithelial cells. Occasionally, smaller cells called nidi, replacement, or regenerative cells are observed between epithelial cells (1,6).

Research on spiroplasma transmission by leafhoppers must account for the ability of the pathogen to traverse the physical barriers (gut wall and salivary glands) within the insect. Before the movement of the pathogen S. citri can be understood, the ultrastructure of the leafhopper barriers must be described. Liu et al. (5) observed S. citri in the gut lumen of C. tenellus, but they did not identify the region of the gut through which the spiroplasmas passed into the hemolymph. Viruses have been reported to accumulate and possibly to multiply in the midgut of leafhoppers (7). Since the lumens of both the foregut and hindgut of C.

tenellus are lined by a thin cuticle, which could block the passage of pathogens from the lumen into the hemocoel, and since the midgut is the active site of nutrient uptake, it is likely that the midgut is the region of passage for spiroplasmas. Therefore, the objective of this work was to describe the morphology and ultrastructure of the different regions of the midgut of C. tenellus.

MATERIALS AND METHODS

Tissue preparation. C. tenellus leafhoppers were maintained on sugarbeet (Beta vulgaris L., cv. 'Giant Western') plants in a growth chamber with a 14 day/10 night photoperiod at 27±1 C. Adult leafhoppers were immobilized in wax and their heads excised. The midguts were removed into a droplet of fixative (4% paraformaldehyde/0.1% glutaraldehyde in 0.1M potassium phosphate buffer, pH 7.2) and fixed for 2 hr at room temperature. The samples were washed in 0.1M phosphate buffer three times for 20 min each and post-fixed in 1:1 2% osmium tetroxide/0.2M potassium phosphate buffer for 2 hr at room temperature. The tissues were washed in 0.1M phosphate buffer three times for 20 min each and enbloc stained in 0.5% aqueous uranyl acetate (UA) overnight at room temperature. The midguts were dehydrated in a graded ethanol series (50, 70, 90, 95, 100, 100, 100%) for 20 min each at room temperature and washed in 100% propylene oxide three times for 20 min each. The samples

were infiltrated in 1:1 propylene oxide/Polybed 812 (21 ml Polybed, 13 ml dodecenylsuccinic anhydride, 11 ml nadic methyl anhydride, 0.7 ml 2,4,6-tri (dimethylaminomethyl) phenol-30) in capped vials overnight at room temperature. Vials were uncapped for approximately 7 hr in a vacuum desiccator. The midguts were flat-mold embedded in fresh Polybed for 48 hr at 60 C.

Sectioning. Blocks were thick (0.5 um) and thin (0.07 um) sectioned on a Sorvall MT-6000 microtome (Research and Manufacturing Company, Tuscon, Arizona). Thick sections of eleven insects were placed on glass slides, stained with Mallory's Azure II Methylene blue (1% Azure II, 2% Methylene blue, 2% Borax), and examined with an Olympus BH2 light microscope (Hitschfel Instruments, Inc. St. Louis, Missouri). Thin sections from four sites along the meso-midgut and anterior midgut of four insects were placed on 200 mesh nickel grids, post-stained with 5% UA for 4 min and lead citrate (0.03 gm in 10ml water, 2 drops 50% NaOH) for 5 min, and examined with a JEOL 100-CX STEM at 80 kv.

RESULTS

A cross-section of the meso-midgut of C. tenellus is shown in Figure 2. A layer of epithelium lined the midgut lumen, with cells varying in shape and protruding to varying depths (Figures 3,5). Epithelial cells ranged in size from 9x15 um to 24x29 um, were sometimes binucleate, and

contained the typical organelles, i.e., endoplasmic reticulum, ribosomes, and vacuoles. These cells were lined with microvilli which protruded into the lumen (Figures 3,4). The microvilli were approximately 0.1x0.9 um and were covered in places with a thin glycocalyx, or membrane, which separated them from the gut lumen. Moving outward from this layer was a second layer of epithelial cells, and a layer of muscle cells, approximately 1.0 to 1.8 um in thickness. Numerous mitochondria (0.5 to 1.0 um in length) were present in the muscle cells and near the microvilli of epithelial cells. A basal membrane (0.05 um thick) separated the midgut layers from the hemocoel (Figure 3,5).

Though larger in size, the filter chamber (FC), or anterior midgut, had an ultrastructure very similar to that of the meso-midgut (Figure 6). Facing the lumen was a layer of epithelial cells which extended into the lumen to varying depths. Microvilli lined the lumen side of the epithelial cells. A layer of muscle cells lined the other side of epithelial cells, and a basal membrane separated the muscle layer from the hemocoel. Mitochondria were present in both the muscle cells and near the microvilli, as well as being scattered throughout other areas of the epithelial cells.

Rod-shaped bacteria-like organisms (0.5 to 2.6 um in length and 0.3-0.5 um in width) were seen in the meso-midgut and anterior midgut lumens, and in membrane-bound pockets in the epithelial cells, of all (11/11) leafhoppers examined

(Figures 4,5,6).

DISCUSSION

No previous reports have given detailed descriptions of the ultrastructure of the digestive tract, specifically the midgut, of the leafhopper C. tenellus. Liu et al (5) reported the presence of S. citri within the gut lumen of C. tenellus, but they did not describe the gut morphology. Although the epithelial cell micrographs of Liu et al. are not as distinct as the ones presented here, the muscle layer, basement membrane, and microvilli appear similar in structure in the two studies. Cheung and Purcell (4) give a similar description of the ultrastructure of the digestive system of the leafhopper Euscilidius variegatus. The present study was undertaken to provide a better understanding of the internal morphology of C. tenellus, which will facilitate the investigation of pathogen invasion through the gut wall.

The filter chamber (FC), or anterior midgut, was similar in ultrastructure to the meso-midgut. Its function is to shunt water to the hindgut, concentrating the nutrients for uptake in the meso-midgut. Since the epithelial cells of the FC, lined with microvilli, invaginated into the lumen, it is possible that nutrient uptake occurs here as well as in the meso-midgut. Furthermore, since it is likely that the meso-midgut is one

site of uptake for S. citri, it is possible that the FC is another.

Liu et al. (5) noted the presence of bacteria-like organisms in the gut lumen of C. tenellus, and postulated that they were symbiotic bacteria. These may be similar to the bacteria which commonly infect the intestines of other insects (4), including leafhoppers and planthoppers. In the planthopper, Peregrinus maidis, filamentous, rhabdo-like structures, spiked double-membraned structures, and rickettsia-like organisms were found in the salivary glands and other organs, while a pathogenic bacterium, Staphylococcus sciuri, was detected in the midgut (2). Cheung and Purcell (4) compared healthy E. variegatus leafhoppers with leafhoppers congenitally-infected with a parasitic, gram-negative bacterium designated BEV. The digestive tracts of BEV-infected leafhoppers exhibited slight deterioration. Epithelial cells became swollen with bacteria, cell membranes began to deteriorate, and the thickness of the glycocalyx covering the microvilli was reduced. The gut lumen of healthy E. variegatus leafhoppers harbored unidentified bacteria which may be symbiotic. In another study, Purcell and Suslow (6) found that the transmission rates of a mycoplasma-like organism, SAY strain, were reduced in E. variegatus leafhoppers infected with BEV.

Until this work, it was not known that the leafhoppers

in our C. tenellus colonies harbored bacteria-like organisms (BLOs). These microbes may have a symbiotic or pathogenic relationship with the leafhoppers. Whether or not these BLOs affect the transmission of S. citri by C. tenellus is currently being studied.

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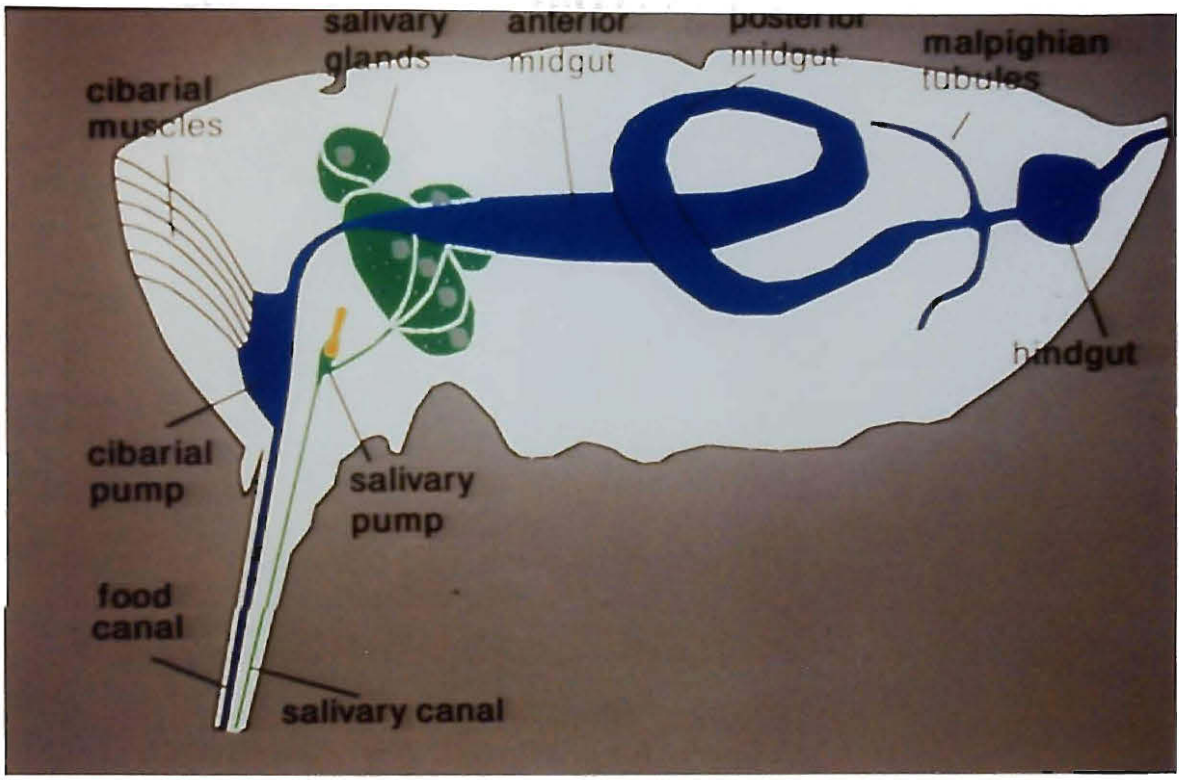

Fig. 1. The internal anatomy of a Cicadellid (courtesy of Astri Wayadande and Jacqueline Fletcher).

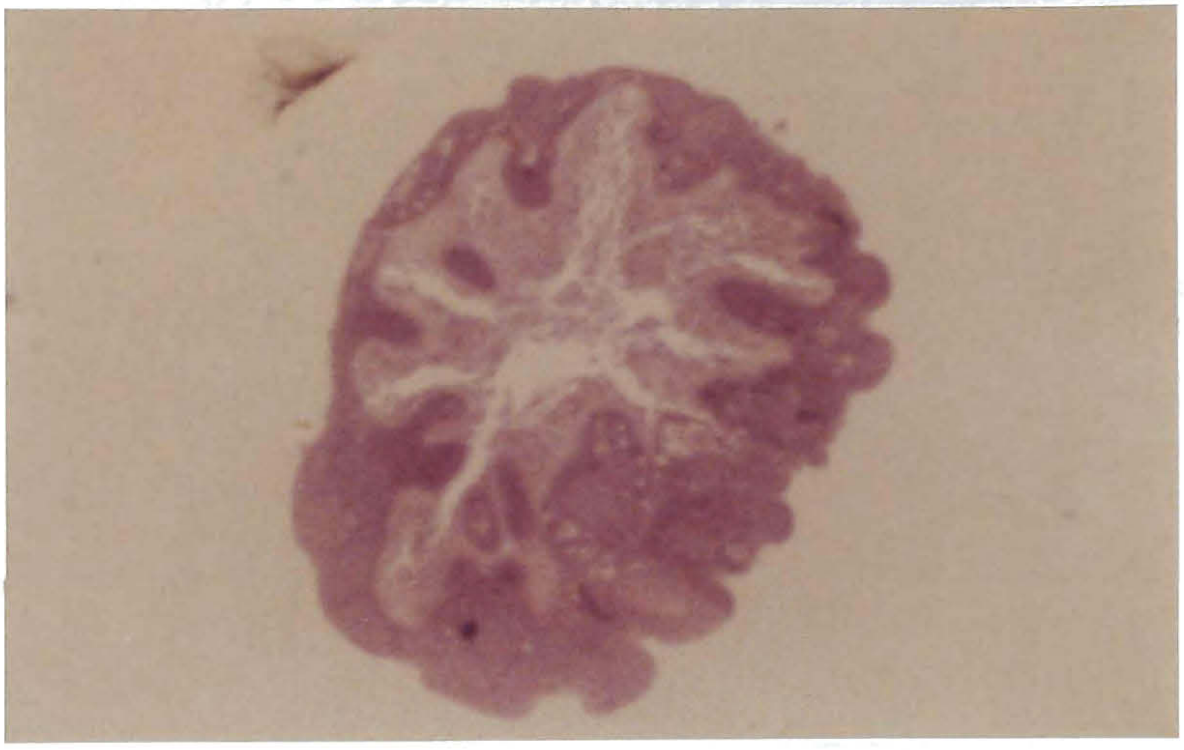

Fig. 2. Light micrograph of a cross section of the midgut of C. tenellus stained with Mallory's stain. 900x.

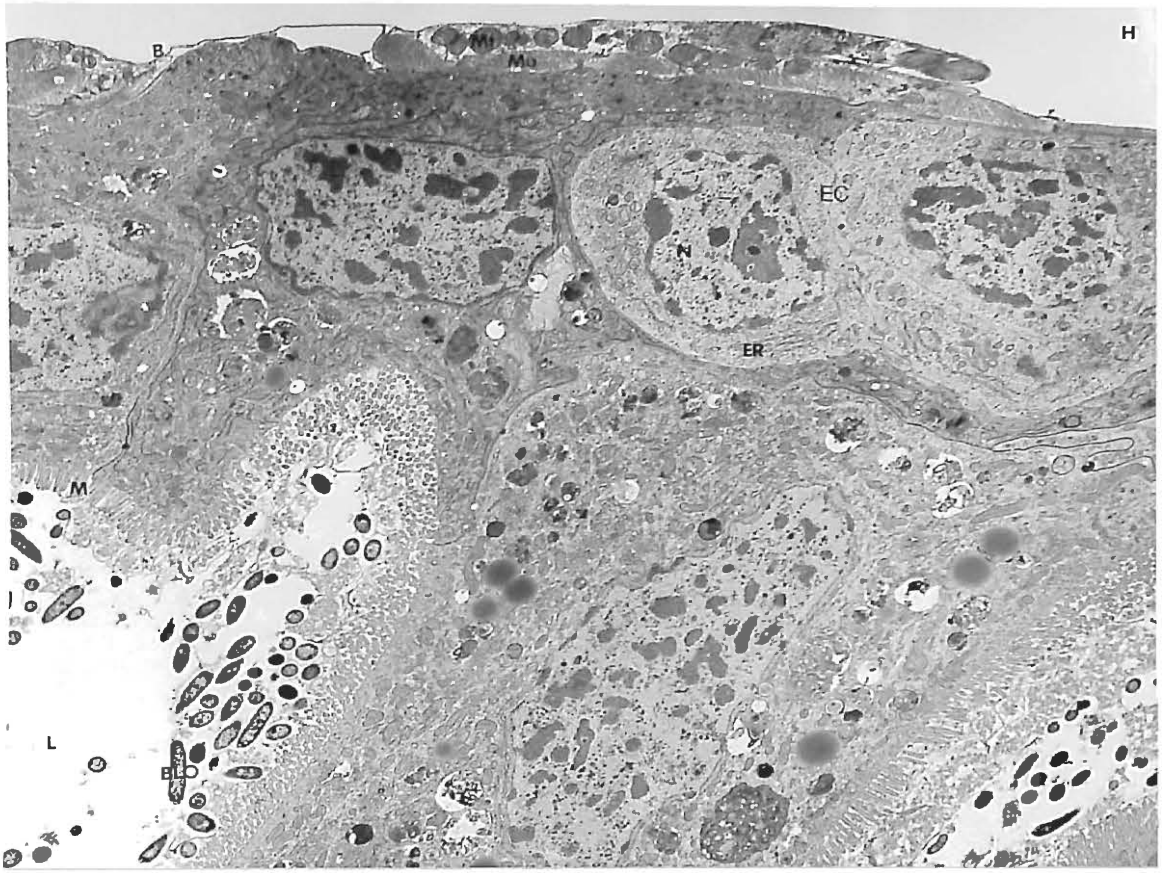

Fig. 3. Electron micrograph of the midgut. L, lumen; H, hemocoel; B, basal membrane; Mu, muscle cell; EC, epithelial cell; N, nucleus of epithelial cell; ER, endoplasmic reticulum; M, microvilli; BLO, bacteria-like organism; Mt, mitochondria. 5000x.

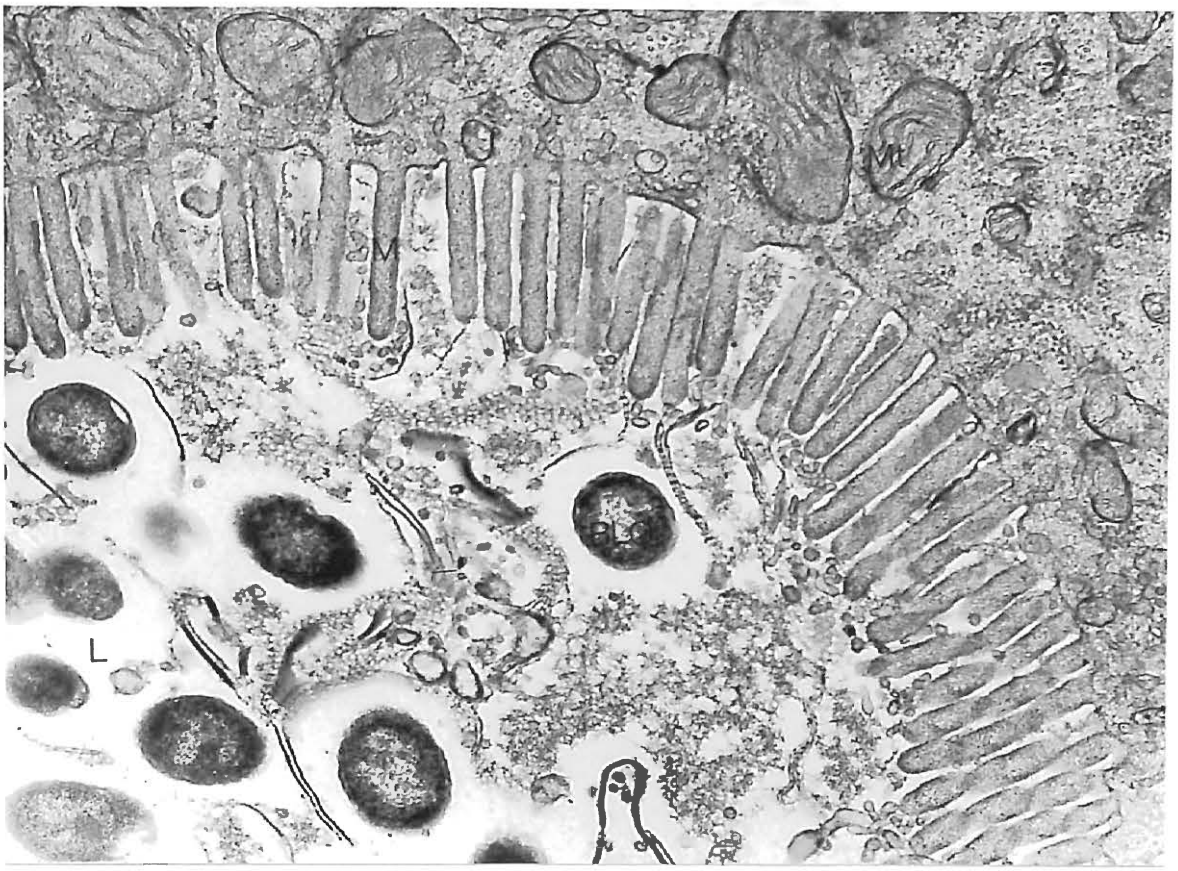

Fig. 4. Electron micrograph of the microvilli (M) lining the lumen side of the epithelial cells. L, lumen; BLO, bacteria-like organism; Mt, mitochondria. 38,000x.

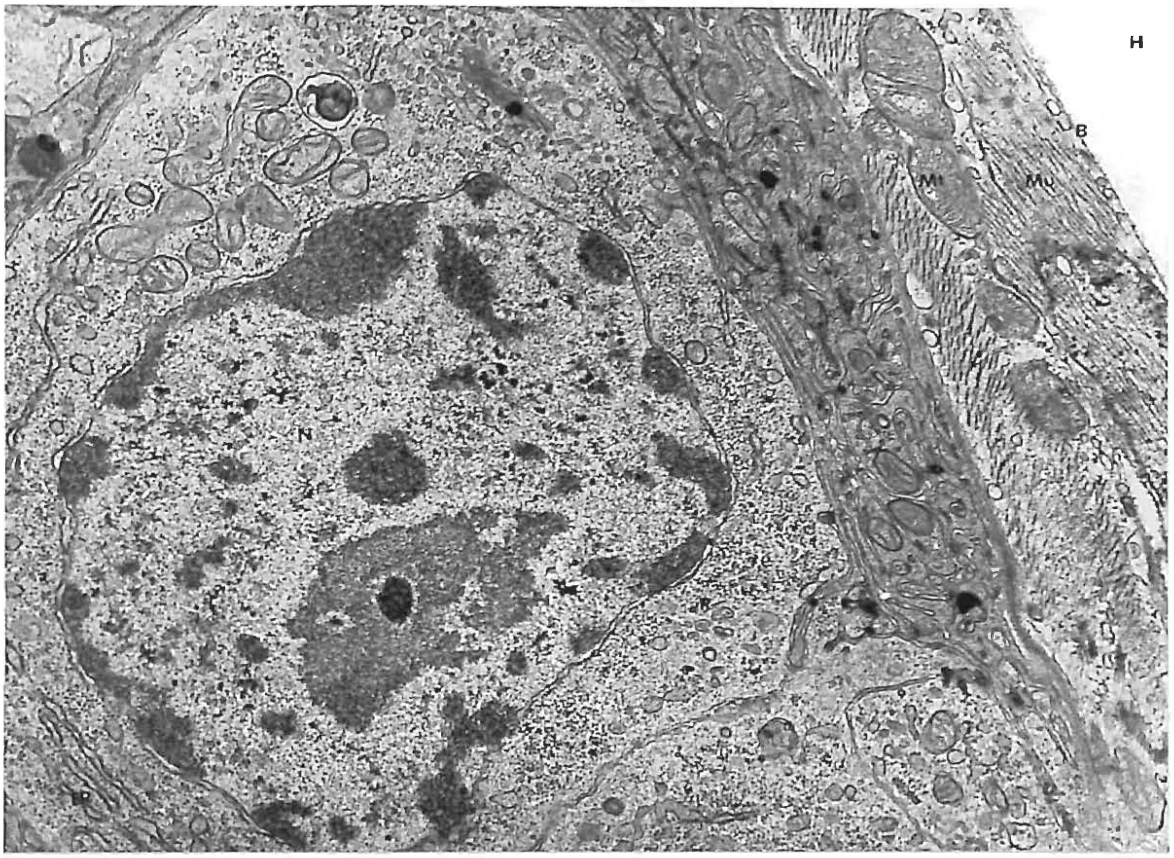

Fig. 5. Electron micrograph of an epithelial cell. H, hemocoel; B, basal membrane; N, nucleus; ER, endoplasmic reticulum; R, ribosomes; Mt, mitochondria; Mu, muscle cell. 20,000x.

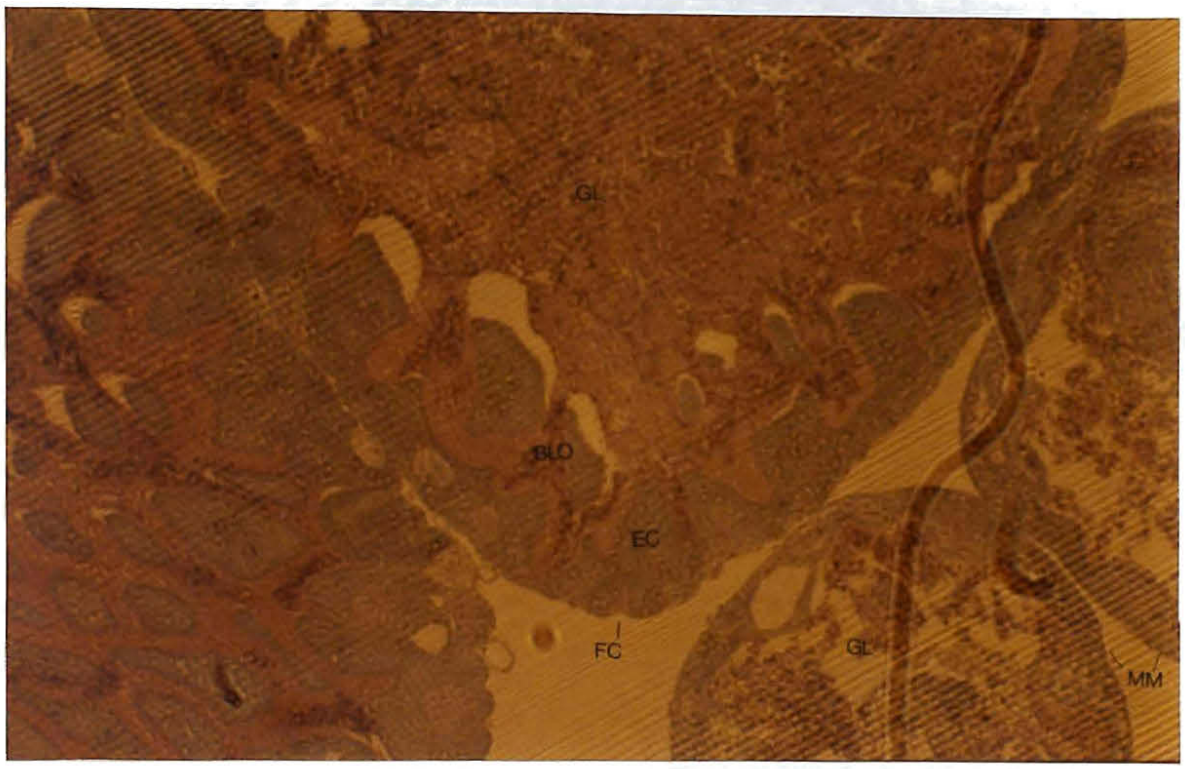

Fig. 6. Light micrograph of a cross section of the meso-midgut and filter chamber of C. tenellus, stained with Mallory's stain. FC, Filter chamber; MM, meso-midgut; EC, epithelial cell; GL, gut lumen; BLO, bacteria-like organisms. 900x.

CHAPTER VII

Immunogold Labeling of *Spiroplasma citri* Within the Midgut of the Leafhopper *Circulifer tenellus*

ABSTRACT

Spiroplasmas have not previously been detected by immunogold labeling within an insect host. Spiroplasmas which have been visualized within insect tissues appear very similar in size and morphology to unidentified bodies present within the insect (i.e. salivary bodies). The purpose of this work was to identify spiroplasmas in both thick and thin leafhopper midgut sections by colloidal gold labeling. Spiroplasmas were detected in *S. citri* exposed leafhoppers but crossreactions occurred in unexposed leafhopper samples and when using preimmune serum.

INTRODUCTION

The life cycle of *S. citri* involves both insect and plant hosts. The major vector of *S. citri* is the beet leafhopper, *C. tenellus* (Baker) (2). Little is known at the molecular level about the interactions of phytopathogenic mollicutes and their leafhopper vectors.

Spiroplasmas are transmitted by leafhoppers in a

propagative manner. Once ingested by the leafhopper, spiroplasmas pass through and multiply in the gut, hemocoel, and salivary glands before being introduced into a new host plant (15). The mechanism of penetration of the gut epithelium and salivary glands of the leafhopper vector C. tenellus by S. citri is unknown. Liu proposed direct penetration and penetration via the endoplasmic reticulum (12). Mowry suggested that S. citri penetrates the barriers of the leafhopper Macrosteles fascifrons by endocytosis (14). Markham (13) postulated that a related spiroplasma, S. kunkelii, enters the gut epithelium and salivary glands via cell junctions, never actually penetrating the cell membrane.

Other insect/pathogen systems have been better characterized. The mechanism of barley yellow dwarf virus (BYDV) penetration of the aphid Rhopalosiphum padi was described, based on electron microscopy, as endocytosis (7). Virions were transported from the hindgut to the hemocoel after specific fusion of the virions with the hindgut basal membrane, followed by receptor mediated endocytosis through the hindgut epithelial plasmalemma. Gildow inferred that penetration of the salivary glands occurs by the same mechanism. The infection of leafhoppers by S. citri may occur by a similar route.

Structural components of the single membrane surrounding mollicutes may allow some of these organisms to

adapt to the host environment through attachment at the cell surface, and may alter host cell functions, which could lead to disease. For example, in the case of Mycoplasma pneumoniae, which causes atypical pneumonia in humans, surface adhesion proteins designated P1 and P30 have been implicated in adherence of the mycoplasma to respiratory epithelial cells (4). The P1 protein is similar both antigenically and in protein sequence, but has limited DNA homology, to a protein of M. genitalium (the pathogen causing nongonoccal urethritis) that is involved in adherence to the urinary tract (3,5). Both of these organisms require the proteins for binding and recognition. It is possible that such proteins exist in spiroplasmas and that they are involved in attachment to physical barriers within the vector.

The present study was part of a larger project to examine transmission specificity of S. citri by the leafhopper C. tenellus. Longterm goals include determination of how spiroplasmas traverse the physical barriers (gut wall and salivary glands) within the insect, and whether it is pathogenicity and/or transmissibility of spiroplasmas that is lost as the cells are repeatedly passed both in vivo and in vitro. Due to difficulty in distinguishing between spiroplasmas and other round bodies in the insect, the specific objective of this work was to label S. citri within the embedding medium LR White and

insect tissues as part of the optimization of electron microscopy procedures necessary for ultrastructural analysis.

MATERIALS AND METHODS

Leafhopper maintenance. All experiments were carried out using C. tenellus leafhoppers maintained on 6-8 wk old sugarbeet plants (Beta vulgaris L., 'Giant Western') in mesh-covered cages (10.75" x 18" x 18") in growth chambers held at 26 C with a 14:10 photoperiod. Approximately 150 fourth and fifth instar nymphs were exposed to S. citri BR3-T in groups of 20-25 in membrane-feeding sachets for an acquisition access period of approximately 24 hr at room temperature and under normal room lighting. Insects then were processed for electron microscopy (EM).

Spiroplasma maintenance. All experiments were carried out using a line of S. citri BR3-3X, originally isolated from Illinois horseradish affected with brittle root disease (8). This line, designated BR3-T, was freshly isolated prior to these experiments from turnip, Brassica rapa L. ('Purple White Top Globe') and allowed to reach log phase in LD8 broth (10) incubated at 31 C. For membrane-feeding experiments, spiroplasmas (1 ml at approximately 10^8 cells/ml) were pelleted at 2940-5220 g at 22 C for 2 min and resuspended in 400 ul of D10 feeding solution (1) (10% sucrose, 0.2% fructose, 0.38% potassium phosphate, 0.03%

magnesium chloride, 1% fetal bovine serum, pH 7.0; filtersterilized).

Lowicryl experiments. Samples processed for EM included (i) C. tenellus nymphs membrane-fed BR3-T in D10 for 24 hr; (ii) C. tenellus nymphs membrane-fed BR3-T in D10 for 24 hr and then caged on sugarbeet for 24 days in a growth chamber at 26 C with a 14:10 photoperiod; (iii) C. tenellus nymphs membrane-fed D10 without spiroplasmas for 24 hr; (iv) C. tenellus nymphs membrane-fed BR3-T in D10 for 18 hrs; and (v) S. citri line BR3-T at 10^8 cells/ml in LD8 broth.

LR White experiments. Samples processed for EM included (i) C. tenellus nymphs membrane-fed 24-48 hr on BR3-T in D10 and then caged on sugarbeet in a growth chamber at 26 C with a 14:10 photoperiod for 5 weeks; (ii) C. tenellus nymphs membrane-fed D10 without spiroplasmas for 24 hr; and (iii) S. citri line BR3-T at 10^8 cells/ml in LD8 broth.

Electron Microscopy processing.

Lowicryl experiments. Leafhoppers for samples i, ii, iii, and iv were immobilized on the surface of a layer of wax and covered with a droplet of fixative (4% paraformaldehyde, 0.1% glutaraldehyde, 0.1M phosphate buffered saline (0.1M phosphate buffer, 0.9% sodium chloride, pH 7.2 (PBS)). The heads were removed and the intestines excised. For sample v, 1 ml of S. citri BR3-T

(approximately 10^8 cells/ml) was centrifuged in an Eppendorf tube at 2940-5220 g and pellets were embedded in 50 ul 1% agarose. All samples were placed in fixative for 4 hr at room temperature, rinsed 3 times in 0.1M PBS for 20 min, and left overnight at 4 C.

Samples were dehydrated in a graded ethanol (EtOH) series in an aluminum block holder on a rocker: 30% EtOH on ice for 30 min; 50% EtOH on 3:1 ice:salt at 4 C for 50 min; 70%, 95%, 100%, 100% EtOH at -20 C for 50 min each; 1:1 100% EtOH:Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, PA; components mixed according to kit directions) at -20 C for 50 min; 1:2 100% EtOH:Lowicryl at -20 C for 50 min; 100% Lowicryl three times for 50 min each and fresh Lowicryl overnight at -20 C. Samples were embedded by placing them in 1 ml gelatin capsules (EMS, size 00) containing approximately 1 ml Lowicryl embedding medium at -20 for 24 hr under UV light (15 watts, 360 nm wavelength, 30-40 cm from sample) and then at room temperature for 48 hr.

LR White experiments. Leafhoppers for samples i and ii were immobilized on the surface of a layer of wax and covered in a droplet of fixative (2% paraformaldehyde, 0.5% glutaraldehyde, 0.1M PBS). The heads were removed and the intestines excised. For sample iii, 1 ml S. citri BR3-T culture (approximately 10^8 cells/ml) was centrifuged in an Eppendorf tube at 2940-5220 g and pellets were embedded in

50 ul 1% agarose.

All samples were placed in fixative for 4 hr at room temperature, rinsed 3 times in 0.1M PBS for 20 min each, and left overnight at 4 C. Samples were dehydrated in 50% and 70% EtOH for 20 min each and then incubated in 2:1 LR White (EMS, Fort Washington, PA):70% EtOH for 1 hr at room temperature in a desiccator. Tissues were placed in 100% LR White overnight at room temperature in a desiccator, transferred to fresh LR White for 1 hr, and then embedded by placing samples in gelatin capsules (EMS, size 00) containing approximately 1 ml LR White. The capsules were placed in a 50 C oven for one wk in one experiment and in a 39 C oven for one wk in another experiment.

Sectioning. Blocks were sectioned with a Sorvall MT6000 ultramicrotome (Research and Manufacturing Company, Tucson, AZ). Thick sections (0.5 um) were cut using a glass knife and placed on glass slides and thin sections (0.07 um) were cut with a diamond knife and placed on 200 mesh nickel grids.

Staining and labeling.

Lowicryl experiments. Using the protocol of Lherminier et al. (11) thick sections were blocked 30 min in BSA (1% in TBS (10 mM Tris-HCl, 40 mM NaCl, 0.05% Tween)). Sections were rinsed three times in TBS and incubated in antiserum against S. citri whole cells (9) (1:100 in TBS) or in preimmune serum (1:100 in TBS) overnight at 4 C.

Sections were rinsed in TBS, incubated in goat anti-rabbit 10 nm colloidal gold (Sigma, St. Louis, MO, 1:20 in TBS), rinsed 2 times in TBS, and incubated in TBS for 3-4 min.

Thick sections were developed with a Silver Enhancement kit (Sigma, components mixed according to kit directions) for approximately 10 min, rinsed quickly with distilled water and soaked in 2.5% sodium thiosulfate pentahydrate for 5 min. Slides were rinsed with distilled water and examined with an Olympus BH-2 light microscope (Hitschfel Instruments, Inc. St. Louis, MO). Slides were counter-stained with Dienes' stain (6). Thin sections were post-stained with 5% aqueous uranyl acetate for 4 min and examined with a JEOL-100 CX STEM at 80 kV.

LR White experiments. Sections were blocked 30 min in BSA (1% in PBS containing 0.05% Tween (PBS-T)). Sections were rinsed four times in PBS-T and then incubated in antiserum against S. citri whole cells (1:100 in PBS-T), preimmune serum (1:100 in PBS-T), or PBS-T only at 4 C for 3.5 hr. Sections were rinsed six times in PBS-T, incubated in goat anti-rabbit colloidal gold diluted 1:20 in PBS-T for 1 hr at room temperature, and rinsed six times with PBS-T.

Thick sections were developed with the Silver Enhancement process and counterstained as described for the Lowicryl experiments. Thin sections were post-stained with 5% aqueous uranyl acetate for 4 min and examined with a JEOL-100 CX STEM at 80 kV.

RESULTS

Diffuse stain, indicative of the collodial gold label, were seen in one experiment in thick sections of three spiroplasma-fed leafhoppers embedded in Lowicryl, incubated in antiserum against S. citri whole cells, silver-enhanced, and counter-stained with Dienes' stain (Figures 1 and 2) suggesting the presence of spiroplasmas. Significant staining was observed in the midgut, including filter chamber and meso-midgut, while the malpighian tubules did not stain. Sections of three spiroplasma-fed leafhoppers incubated in preimmune serum exhibited only faint or no labeling. Sections of two leafhoppers membrane-fed D10 without spiroplasmas and incubated in antiserum against S. citri whole cells and preimmune serum exhibited only faint or no labeling (Figures 3 and 4). BR3-T cells embedded in Lowicryl did not become well-enough infiltrated for sections to be obtained. Specific labeling was observed in only one Lowicryl experiment, however, and two repeated attempts at labeling sections of the leafhoppers mentioned above resulted in non-specific labeling. In another Lowicryl embedding experiment, gold particles were seen in tissues of all treatments indicating non-specific labeling.

In three LR White embedding experiments, non-specific labeling was observed in sections of tissues from all treatments.

DISCUSSION

As seen in Figures 1 and 2, specific labeling of the intestines was suggestive of the presence of S. citri line BR3-T in the midgut and filter chamber. However, these results have not been duplicated. In subsequent replications, labeling was observed in leafhoppers not exposed to S. citri, in tissues not believed to harbor spiroplasmas (i.e. Malpighian tubules), and in tissues incubated in preimmune serum.

Due to the difficulty encountered in embedding and sectioning tissues in Lowicryl, and since repeated tries at specific labeling failed, LR White was substituted as the embedding medium. LR White has been recommended for use in immunocytochemistry (11,16). There are many possibilities as to why only non-specific labeling was observed with LR White. In one experiment, samples were embedded at 50 C. Because of concern that this high temperature could affect antigenicity, samples were then embedded at 39 C. However, again only non-specific labeling was observed. It is possible that the sites for antigen recognition may not have been available or that spiroplasmas were not present in the tissues due to the poor transmission rate (10% or lower) of S. citri by C. tenellus under our conditions (A. Wayadande, personal communication). The silver enhancement chemicals could have been contaminated thus resulting in non-specific labeling. Furthermore, the LR White embedding medium may

have been defective due to polymerization problems, however, samples did polymerize and were suitable for sectioning.

In both Lowicryl and LR White resins, the samples of S. citri cells alone were never properly embedded, so sections could not be obtained. This meant there was never a "positive" control. It is possible that the dilution of antiserum may not have been appropriate, which could have resulted in little to no labeling. Sections may have been over-rinsed so that all the protein A-gold was rinsed away, or under-rinsed which could result in non-specific labeling.

No previous reports have positively identified spiroplasmas by immunogold labeling within an insect host. It was hoped that in this work a methodology could be established for a more positive identification of S. citri within the leafhopper vector, C. tenellus, due to the difficulty in distinguishing S. citri from other round bodies in the insect. Unfortunately, this objective was not accomplished.

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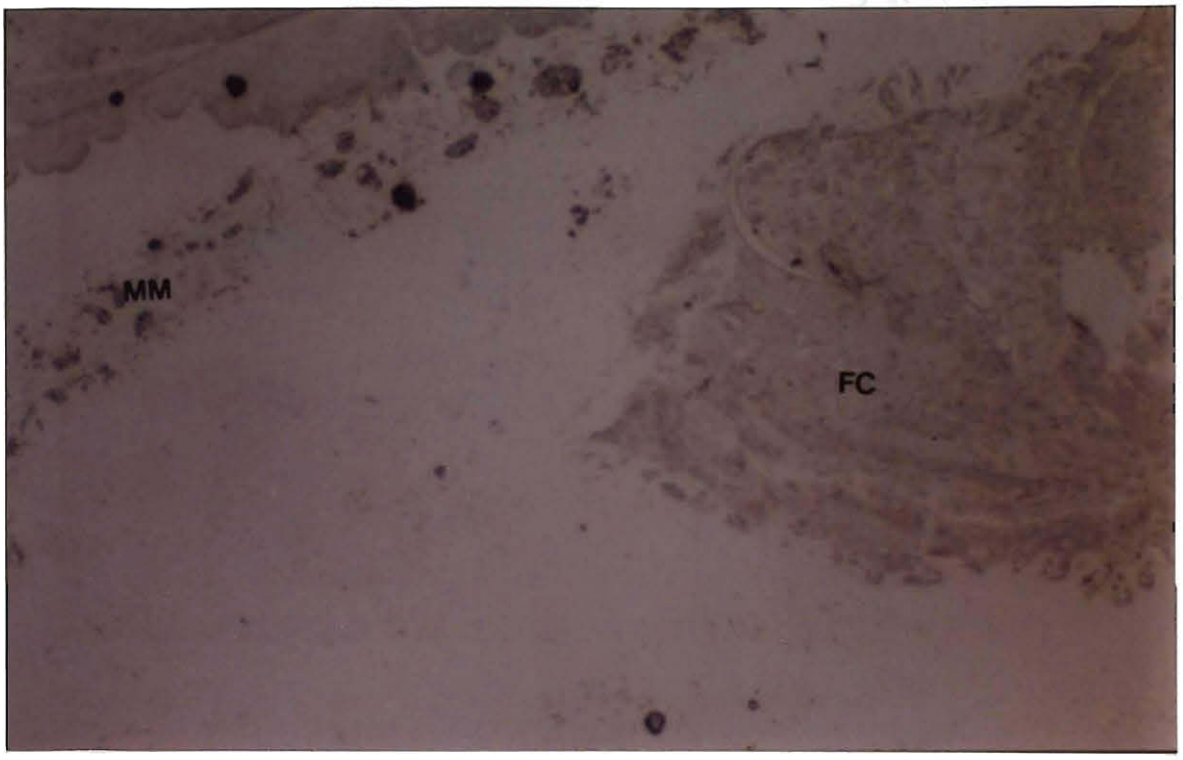

Fig. 1. Light micrograph of a cross-section of the midgut from a spiroplasma-fed C. tenellus leafhopper embedded in Lowicryl and silver-enhanced. 800x. (FC, filter chamber; MM, meso-midgut).

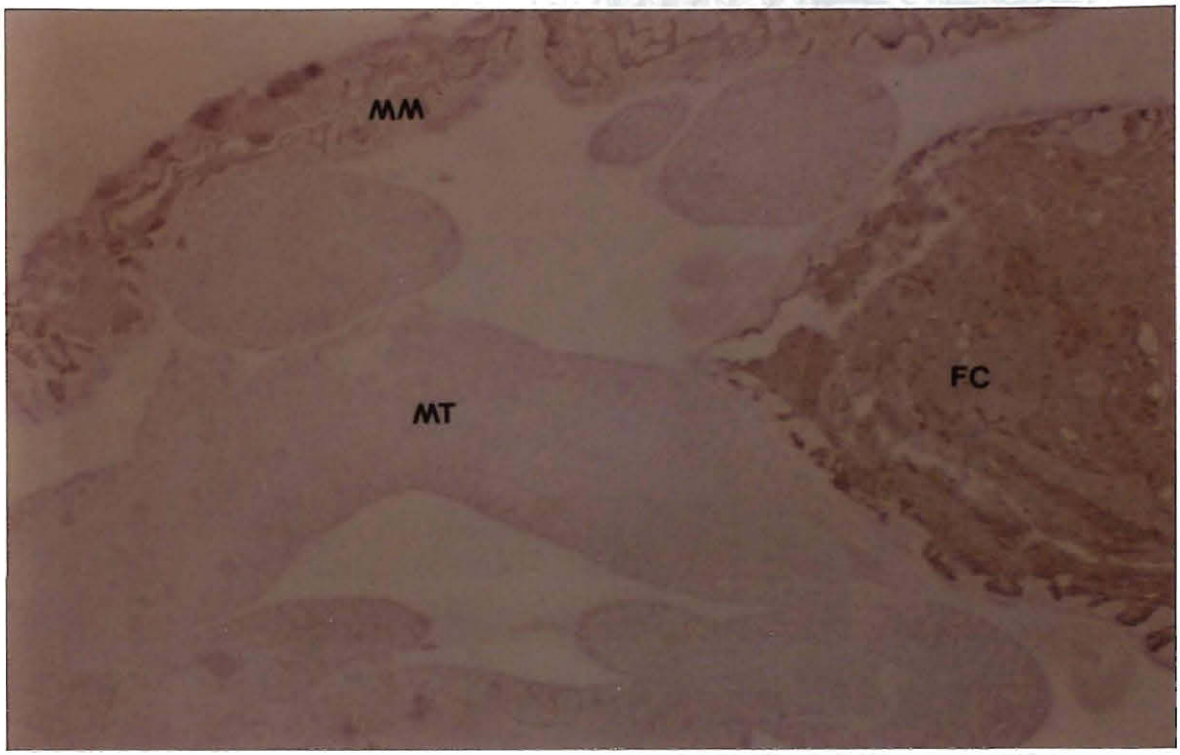

Fig. 2. Light micrograph of a cross-section of the midgut from a spiroplasma-fed C. tenellus leafhopper embedded in Lowicryl, silver-enhanced (denser regions), and Dienes' stained. 700x. (FC, filter chamber; MT, Malpighian tubules; MM, meso-midgut).

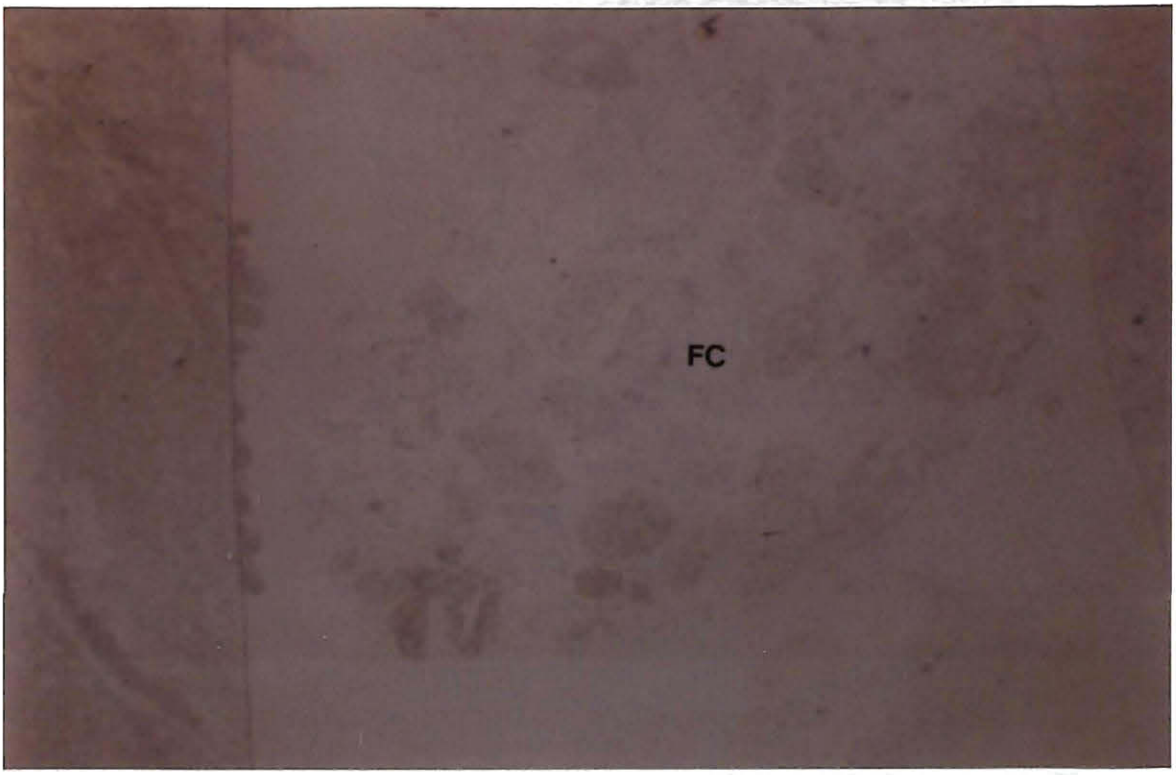

Fig. 3. Light micrograph of a cross-section of the midgut from a leafhopper membrane-fed D10 without spiroplasmas embedded in Lowicryl and silver-enhanced. 800x. (FC, filter chamber)

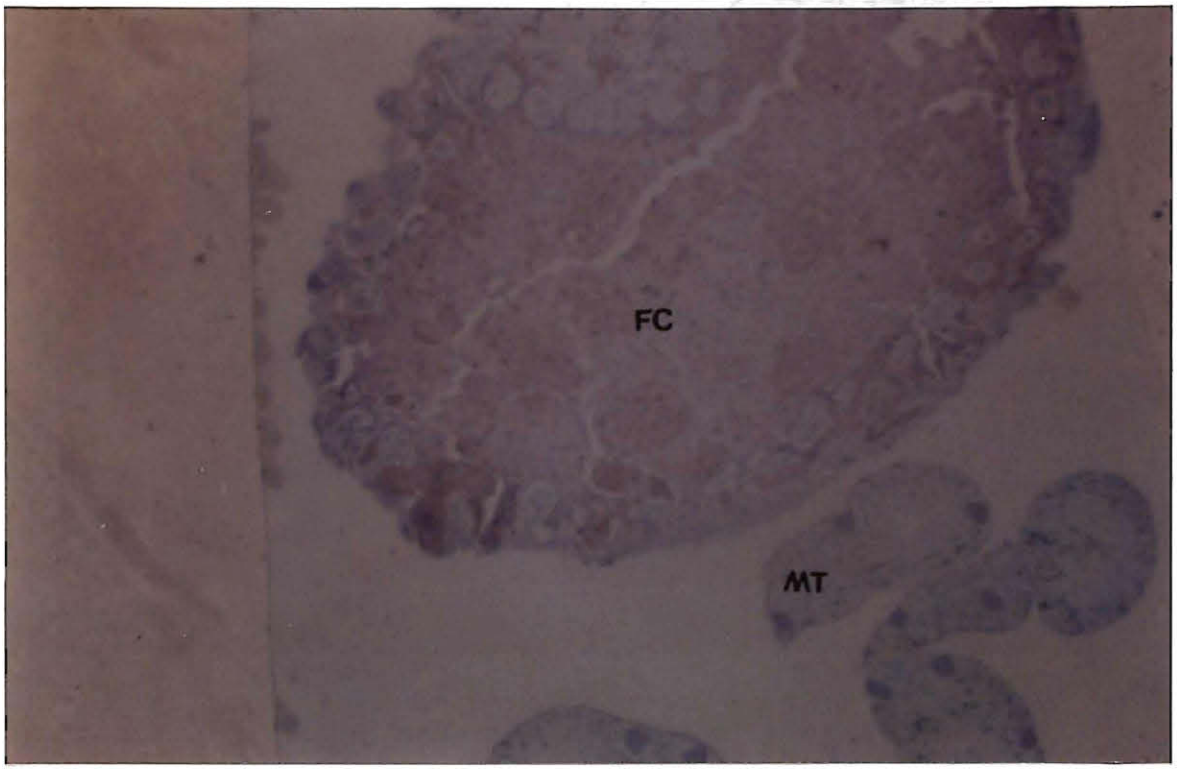

Fig. 4. Light micrograph of a cross-section of the midgut from a leafhopper membrane-fed D10 with spiroplasmas embedded in Lowicryl, silver-enhanced, and Dienes' stained. 900x. (FC, filter chamber; MT, Malpighian tubules).

VITA

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MASTER OF SCIENCE

Thesis: EXPRESSION OF SPIROPLASMA CITRI PROTEINS UNDER VARYING IN VITRO CONDITIONS AND THEIR IMPORTANCE IN TRANSMISSION BY THE LEAFHOPPER CIRCULIFER TENELLUS

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